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Parasitology

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Životní cyklus volně žijících améb

Diferenciace amfizoických améb rodu *Acanthamoeba* a *Balamuthia*

Life cycle of the free-living amoeba

Differentiation of amphizoic amoebae of the genera *Acanthamoeba and Balamuthia*

Ph.D. Thesis

Supervisor: RNDr. Eva Nohýnková, PhD

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Motto: Don´t panic.
Douglas Adams, 1986

Abstract:

Free-living pathogenic amoebae *Acanthamoeba* spp. and *Balamuthia mandrillaris* are causative agents of important diseases of human: rarely occurring but highly fatal granulomatous amoebic encephalitis (both) and keratitis (*Acanthamoeba*). One of the reasons for the problematic therapy is differentiation into highly resistant cysts often found in affected tissues. In our study we have found that correct encystation in *Acanthamoeba* requires apart from others, the presence of functioning Golgi apparatus transporting the cyst wall material to the cell surface; glycogen phosphorylase degrading glycogen into glucose which seems to be further used for cellulose synthesis and two non-constitutive cellulose synthases. *Acanthamoeba* cellulose synthases seem not to be inhibited by known herbicides. In the cyst wall of acanthamoebae we detected cellulose, β-mannan, and β-1, 3-1, 4-linked glucan [lichenin or mixed-linkage glucan (MLG)]. Cellulose is present in the inner (endocyst) and the outer (exocyst) layers of the cyst wall, whereas β-mannan and MLG are found in the endocyst. In a protozoan organism, MLG was detected for the first time. The MLG of *Acanthamoeba* has a similar composition to that found in barley with high amount of cellobiosyl and cellotriosyl followed by cellotetraosyl units. In contrast, with the same approach we do not detect any polysaccharides or carbohydrate moieties in *Balamuthia* cyst walls. Instead, using proteases with distinct specificities we found out that the cyst walls of balamuthias are mostly composed of cysteine-rich proteins. Cellular debris in form of lipid granules was also detected. Further we found that beside cysts, acanthamoebae are able to differentiate into a distinct dormant stage, which we described as a pseudocyst. Formation of the pseudocyst occurs as a prompt reaction to treatment with organic solvents, namely methanol, dimethylsuphoxide, or acetone. The pseudocyst differs from the mature cyst in velocity of formation (2 hours post-treatment), architecture of cell envelope (surface coat resembling glycocalyx), and carbohydrate composition of the envelope (mainly glucose subunits). The pseudocyst coat protects the cell from alkaline pH and higher temperatures. Moreover, we found that also propylene glycol (PG) and contact lens solutions containing PG induce the pseudocyst formation. It seems that chronic stressful conditions such as starvation induce differentiation of acanthamoebae into a cyst, whereas acute toxic changes of the environment, especially the presence of substances, which can interfere with plasma membrane (or propylene glycol), lead to the pseudocyst formation.

Abstrakt:

Volně žijící améby *Acanthamoeba* spp. a *Balamuthia mandrillaris* jsou původci závažných onemocnění člověka: vzácné, většinou fatální granulomatózní amébové encefalitidy (obě) nebo keratitidy (akantaméby). Jedním z důvodů neúspěšné terapie je schopnost trofozoitů obou organizmů vytvářet rezistentní stádia - cysty, které lze najít také v postižených tkáních. V naší práci jsme zjistili, že encystace u akantaméb vyžaduje kromě jiného přítomnost fungujícího Golgiho aparátu, který se podílí na trasportu materiálu pro stavbu cystové stěny na povrch buňky; glykogenfosforylázy, enzymu, který degraduje glykogen na podjednotky glukózy využívané encystující buňkou pro syntézu celulózy a dvě nově exprimované celulóza syntázy. Syntéza celulózy u akantaméb není inhibovaná známými herbicidy. Kromě celulózy jsme v cystové stěně akantaméb detekovali β-mannan a β-1,3-1,4-glukan (lichenin). Zatímco celulóza je přítomna ve vnitřní (endocystě) a zevní stěně (exocystě) cysty, β-mannan a lichenin se nacházejí pouze v endocystě. Lichenin, který byl u protozoí v naší studii detekován vůbec poprvé, má podobnou stavbu jako lichenin ječmene. Je tvořen především celobiózou a celotriózou s menším podílem celotetrózy. Použitím stejných metod jako u akantaméb se nám nepodařilo detekovat žádný cukerný zbytek v cystové stěně balamutií. Naopak, pomocí specifických proteáz jsme zjistili, že *B. mandrillaris* má cystovou stěnu složenou zejména z proteinů s vysokým podílem cysteinu. V cystové stěně obou améb jsme také detekovali buněčný odpad, zejména lipidová granula. Popsali jsme dosud nepopsaný způsob diferenciace akantaméb za vzniku dormantního stádia, které jsme nazvali pseudocysta. Tvorba pseudocyst je rychlou odpovědí na expozici akantaméb vůči organickým rozpouštědlům: metanolu, dimetylsulfoxidu nebo acetonu. Pseudocysty se liší od zralých cyst rychlostí tvorby (2 hodiny), architekturou povrchové struktury (plášť připomínající glykokalyx) a složením pláště (glukózové/manózové podjednotky). Pseudocystový plášť chrání buňku před alkalickým pH a vyšší teplotou. Dále jsme zjistili, že propylenglykol (PG) a roztoky na čištění kontaktních čoček, které PG obsahují, také indukují tvorbu pseudocyst. Z našich výsledků plyne, že chronické nepříznivé podmínky vnějšího prostředí jako hladovění indukují encystaci, zatímco akutní změny prostřední, zejména přítomnost látek, které mohou poškodit plasmatickou membránu (nebo propylenglykol), vedou k tvorbě pseudocyst.

Content:

List of terms and abbreviations

AK – *Acanthamoeba* keratitis
ARF - adenosyl-ribosylation factor ATP – adenosyltriphosphate
BAE – *Balamuthia* granulomatous amoebic encephalitis
BFDA – Brefeldin A
cAMP – cyclic adenosinemonophosphate
CesA – cellulose synthase enzyme
CNS – central nervous system
ConA – Concanamycin A
COPI – Golgi-derived coated vesicles
Csl – cellulose synthase-like enzymes
CSP21 – cyst specific protein 21
CW – cell wall or cyst wall in respective organisms
DB – degree of polymerisation
DCB – dichlorobenzonitrile
DcsA – *Dictyostelium* cellulose synthase A gene
DM – diabetes mellitus
DMSO - dimethylsulphoxide
DP – degree of polymerisation
EM Neff – encystation medium according to Neff
ER – endoplasmic reticulum
GAE – *Acanthamoeba* granulomatous amoebic encephalitis
GCA - 1-cyclohexyl-5-(2,3,4,5,6-pentafluorophenoxy)-1lambda4,2,46-thiatriazin-3-amine
GT – glycosyltransferase
GTP - guanosyltriphosphate
HBMEC – human brain microvascular endothelial cells
HIV – human immunodeficiency virus
MLG – mixed linkage glucan (lichenin or β-1,3-1,4-glucan)
PAS – periodic Acid Schiff staining
PHMB - polyhexamethylene biguanide
PI – propidium iodide
RNA – ribonucleic acid
sIgA – secretory immunoglubulin A
SNAP – N-ethylmaleimide-sensitive attachment protein
SNARE – SNAP receptors
TCA – tricarboxylic acid cycle
TGN – trans Golgi network
UDP – uridine diphosphate
UV - ultraviolet

1. Introduction:

1.1 Differentiation:

Differentiation is a process in which a cell gains distinct characteristics enabling it to adapt to a specific environment. During the process the cell starts to express a unique combination of genes/proteins and whole constellation of its characteristics change in a specific sequence leading to a cell with exact morphology, behaviour and function (Fulton, 1977). Differentiation can describe the transformation of e.g., pluripotent stem cells into different tissue cells, or conversion of unicellular eukaryotes (protists) into different stages during their life cycles (Chavez-Munguia et al., 2007; Hindley and Philpott, 2013). Formation of resistant, dormant forms such as cysts or spores enables free-living protists to withstand the external conditions, which are not favourable for growth and multiplication or are even harmful. It is be essential for transmission of cyst-forming protozoan parasites such as *Giardia intestinalis, Entamoeba histolytica* or *Toxoplasma gondii* to a new host when the resistant stages are excreted by infected person or animals into the environment (Lujan et al., 1997; Siddiqui et al., 2013).

The formation of stages, which resist unfavourable conditions, is accompanied by assembly of protective cell envelopes, namely cell walls (CW) (West, 2003; Doering et al., 2009). Similar CWs represent integral part of cells of plants and fungi (Feofilova, 2010; Burton et al., 2010).

My work deals with differentiation of pathogenic free-living amoebae into resistant forms.

The first part of the thesis summarizes current knowledge on differentiation in unicellular eukaryotic organisms and processes of synthesis, assembly and composition of the cyst/spore/plant CW.

The second part is focusing on the current knowledge about the amphizoic amoebae *Acanthamoeba* spp. and *Balamuthia mandrillaris* with special emphasis on their differentiation into the dormant cysts.

Generally, the differentiation can be divided into three phases: (1) stimulus leading to regulation of specific genes, (2) synthesis and transportation of specific materials to the cell surface and finally (3) assembly of CW (Lujan et al., 1997).

1.2 Induction of differentiation

To differentiate, a specific signal from the environment is necessary otherwise the cells would continue to grow and multiply (Fulton, 1977). Environmental stresses represented by changes in pH, temperature, osmolarity, redox potential, the presence of specific molecules/ions have been found to lead to conformational and topological changes in sensor molecules located on the cell surface which are subsequently transmitting the signal into the interior of cell (Lujan et al., 1997).

Lack of food/starvation can represent a condition of the environment, which is lacking specific chemical signals released by the bacteria or other organisms ('food source'). It is the lack of chemical signals to which many protists such as *Acanthamoeba* spp., *B. mandrillaris, Naegleria* spp., *Dictyostelium discoideu*m respond by encystation (Chavez-Munguia et al., 2007; West et al., 2008). Starving cells of social amoeba *D. discoideum* are releasing signalling factor also called conditioned medium factor. When its concentration rises above threshold, cells start aggregating with relayed pulses of cyclic AMP (cAMP) acting as chemoattractant and regulator of many genes specific for early development (Jang and Gomer, 2005). Differentiation of *Giardia intestinalis* could be a response to the cholesterol deprivation or presence of bile salts or bile. It is hypothesized that the cholesterol deprivation could induce changes in the fluidity of the *Giardia* membrane leading to the regulation of encystation specific genes, or this regulation might be result of direct gene mediation by cholesterol (Lujan et al., 1997).

However, it seems that the induction of differentiation does not work as a simple switch on and off system as single exposure to the signal results in the arrest of the cell division for some time followed by reentering into the log phase of the growth and only prolonged stimulation results in the encystation (Yang and Villemez, 1994).

1.3 Composition of the cell wall

Cell surface structure composition corresponds to its role and is often very complicated. In plants the CWs are integral part of the cell, therefore they must provide structural support and a protective barrier against osmotic or other physical stress, but at the same time allow the expansion and growth of the cell. Similarly, spores or cyst walls providing the cell with

protection against different unfavourable environmental conditions must allow detection of external signals, informing the organism about favourable conditions and thus allowing exsporulation or excystment.
The composition of the CW structures ranges from completely proteinaceous to completely polysaccharide (Metcalf et al. 2007).

Becker (2000) proposed a classification scheme employing five basic types of the cell surface or CW:

- type I: simple, albeit externally extended, plasma membrane types (glycocalyx of the archebacteria);
- type II: cell surfaces bound to internal materials (such as pellicle of euglenoids);
- type III: surfaces with external materials (CW of land plants and majority of algae);
- type IV: surfaces with internal vesiculated materials;
- type V: surfaces with materials both internal and external to the plasma membrane (periplast in cryptomonads) (Niklas, 2004).

Usually, different amount of carbohydrates and proteins are the main components of the CW in different organisms. While carbohydrates provide the CW with structural integrity, proteins are participating in protein-protein interactions, they crosslink the glycans and/or participate in further modification of carbohydrates or proteins.

1.3.1 Fungi

Surface cell structures in fungi play a multifunctional role: provide the cells with strength and rigidity, maintain the cell shape and regulate the morphogenesis, participate in fungal reproduction, determine the antigenic and adhesive characteristics, control over dimorphism and formation of spores, regulate permeability, control reception of an external signal and its transfer to the membrane and intracellular messenger (Doering et al., 2009, Feofilova, 2010).
Currently, fungal CW components are subdivided into structural components [chitin, β(1–3)–β(1–6)-glucans, and β(1–4)-glucan (cellulose)] and matrix, which includes mannoproteins, galactomannoproteins, xylomannoproteins, glucuronmannoproteins, and α(1–3)-glucan. The

efficiency of the protection against unfavourable conditions varies depending on CW composition, where chitin content is of particular importance.

Chitin (poly-N-acetyl-D-glucosamine) bound to 1,3-glucan is the main part of the fungal wall skeleton. It has got very similar physicochemical properties as cellulose and is abundant especially in starving cells differentiating into spores (Feofilova, 2010).

In typical fungal CW, glucans, such as β-1,3-glucan, are located on the cell surface. Below the glucan, the glycoprotein reticulum is situated, followed by a protein layer with chitin microfibrils embedded in. The protein composition of the CW depends on the developmental stage and the environmental conditions. Lipids (up to 3%), and different proteins such as amyloids (structural components), hydrophobins (involved in adherence), uronic acids and melanin (protection from oxidative stress and UV light) were also isolated from the CWs of *Fungi* (Feofilova, 2010).

1.3.2 Protozoa

Cyst/oocyst wall in protozoa shelters the organisms and allows the survival of free-living organisms when the conditions of the environment are not favourable for growth and multiplication. Moreover, extended survival in the contrasting climatic conditions and disinfection processes has a crucial role in the transmission of *Giardia intestinalis*, *Entamoeba histolytica*, *Toxoplasma gondii* and *Cryptosporidium parvum*. The CW represents a highly resistant barrier and plays role in the interaction with organic and inorganic particles governing the transport, retention in the contaminated soil samples or kinetics of the release by waterbed and survival. Though the biochemical composition and molecular architecture of parasitic respective outer walls differ greatly, the surface interactions with the outer environment are similar depending on the chemistry and topography of the macromolecules at the parasite surface, on their hydrophobicity, electric charge and also external physicochemical conditions such as the ionic composition of the surrounding medium and organic contamination (reviewed in Dumetre et al., 2012).

Similarly to the other CWs, protozoan cyst/oocyst walls can have multiple layers which are composed of combination of proteins, glycoproteins, lipids and carbohydrates found in different amounts in each layer. The structural part of the wall is represented by different polymers of carbohydrates (such as chitin = polymer of N-acetylglucosamine in *E. invadens*, polymer of N-acetyl-galactosamine in *G. intestinalis*, cellulose in *D. discoideum* and *Naegleria gruberi*). Apart from carbohydrates the stability of the wall is a result of the

presence of different crosslinking glycoproteins and proteins which are conferring to the cyst resistance (Jacob and Jessie lectins in *E. invadens*, CWPs in *G. intestinalis*, SP85 in *D. discoideum*) or are capable of their modification (chitinase in *E. invadens*, cellulase in *D. discoideum*) (Werth and Kahn, 1967; Fulton, 1977; Frisardi et al., 1991; Lujan et al., 1997; Thomason et al., 1999; Sun et al., 2003; Chavez-Mungia et al, 2007; West et al., 2008; Chatterjee et al., 2009, Fouque et al., 2012).

1.3.3 Plant cell wall

The best studied composition of eukaryotic CWs come from the studies done in different plants which serve as model organisms: *Arabidopsis thaliana, Oryza sativa, Zinnia elegans, Nicotiana tabacum, Hordeum vulgare* and others. Plant cells are surrounded by strong walls which are important for adaptation of the plant to the environmental changes and provide the cells with structural support (Oikawa et al., 2013; Worden et al., 2012). Generally, two types of CWs are found in the plant cells. Primary walls are deposited during the cell growth. They are firm but at the same time extendible to permit the cell expansion. Secondary walls are deposited after the cessation of the growth and confer to the mechanical stability. They are composed of cellulose, hemicellulose and lignin (Reiter, 2002; Vogel, 2008). In the following text, only the primary CWs are discussed in further detail.

Primary CWs of plants are very complex and dynamic structures which depending on the species, cell type and developmental stage consist mainly of cellulose microfibrils embedded in network of polymers including hemicelluloses (mainly xyloglucans), pectins and structural arabinogalactan proteins (Somerville et al., 2004; Anderson et al., 2009; Peaucelle et al., 2012, Worden et al., 2012). Varieties of glucans are bound together to form a very complex matrix in which the load-bearing components are represented by the major two: $Ca^{2+/}$pectate and cellulose/xyloglucan network (Somerville et al., 2004; Peaucelle et al., 2012).

In the CW of grasses (Poales), some algae, fungi and *Equisetum arvense* specific polysaccharide (1,3;1,4)-β-glucan [mixed linkage glucan (MLG)] is found forming a gel like matrix in which the other polysaccharides are embedded. It might not be essential for the CW integrity but could impact wall function and have role in cell expansion. Its role as the secondary storage of glucose was also proposed (Burton and Fincher, 2012). CW proteins (hydroxyproline- and proline-rich glycoproteins and arabino-galactan proteins) can be covalently, very loosely bound to the polysaccharides or can be present in a soluble form (Vogel, 2008). They provide structure and enable modification of the CW in plants especially during the growth (Worden et al., 2012).

1.4 Biosynthesis and assembly of the cell walls

In all organisms synthesizing the CWs similar problems need to be resolved: which molecules are present in the CW, how are they synthesized and assembled, how exactly do these interact together and what is their individual function (Reiss et al, 1992; Gozalbo et al., 1993; Lujan et al., 1997).

The assembly of CWs is highly organised and disrupture of single step in many might affect the whole formation or lead to decreased resistance of the organism: Correct assembly of the spore coat in *Dictyostelium* requires exact timing and deposition of each of different proteins, carbohydrates and cellulose microfibrils and interference with the process results in increased sensitivity of mutants to detergents and also hypertonic stress (Thomason et al., 1999; Zhang et al., 2001; Metcalf et al., 2007; West et al., 2008; Chatterjee et al., 2009).

1.4.1 Organelles participation in the CW synthesis

Most of the components and activities related to the wall synthesis are tightly correlated with the cellular endomembrane system. Proteins and glyproteins acting as enzymes or being structural part of the CWs are synthesized in the endoplasmic reticulum and subsequently are specifically sorted into correct compartments such as plasma membrane, Golgi apparatus and endoplasmic reticulum itself for further modification (Leucci et al., 2006). Correct target compartment is defined by specific amino-terminal leader sequence of the protein, which is removed in endoplasmic reticulum, where protein is further modified by assembly, folding and core-N-glycosylation (Becker and Melkonian, 1996, Geisler et al., 2008). However, majority of glycosylation reactions including biosynthesis of wall polysaccharides (non-cellulosic plant CW polysaccharides except for callose), their modification by addition of side chains, glycosylation of proteins and lipids and assembly of cellulose synthase complexes take place in Golgi apparatus (Becker and Melkonian, 1996; Paredez et al., 2006; Worden et al., 2012; Oikawa et al., 2013). Its cisternae are divided into several subtypes: cis, medial and trans with processing taking place from cis to trans region, and final packaging and sorting by trans Golgi network (Driouich et al., 1993; Geisler et al., 2008; Worden et al., 2012). Golgi positioning, architecture and trafficking depends on the association with and coordination of microtubules and microfilaments, where increasing pH gradient along the secretory pathway is of crucial importance. It is established by functional vacuolar ATPases (V-ATPase) and maintained indirectly by actin filaments. pH gradient not only affects trafficking but is

important for accurate posttranslation protein and lipid modifications, especially N- and O-glycosylations (Egea et al., 2006; Lazaro-Dieguez et al., 2006, Geisler et al., 2008; Rivinoja et al., 2009; Worden et al., 2012).

Transport of substrates, prefinal or final products from ER to Golgi network, between different Golgi cisternae and to the plasma membrane relies on COPI coated, whereas products/substrates aimed for lysosomes are transported by clathrin coated vesicles. Seven coat proteins, adenosyl-ribosylation factor (ARF) and ATP are necessary for the formation of COPI coated vesicles (Becker and Melkonian, 1996).

After reaching the final destination, the vesicles are uncoated and fusion complex consisting of v-SNARE (vesicular membrane proteins) and t-SNARE (target membrane protein) is formed (Becker and Melkonian, 1996). At plasma membrane vesicles release numerous membrane associated proteins including cellulose synthase complexes, CW polysaccharides and structural and enzymatic proteins each in separate vesicles (Leucci et al., 2006).

Golgi complex seems to play crucial role also in the encystment of different protozoa such as *Entamoeba*, *Giardia* or *Dictyostelium* (West et al., 2008). During differentiation, CW material is transported to the cellular surface in encystation specific vesicles, which upon reaching their destination release their content by exocytosis. Encystation specific vesicles seem to be very common features of encysting organisms: they were detected in *G. instestinalis* (containing cyst wall proteins), *E. invadens* (containing fibrillar material positive in Calcofluor white staining) and *Acanthamoeba* spp. (Lujan, 1997; Benchimol, 2004; Chavez-Munguia et al., 2003; Chavez-Munguia et al., 2005; Chavez-Mungia et al., 2007).

1.4.2 Inhibitors of intracellular trafficking

Brefeldin A (BFDA), hydrophobic fungal antibiotic is a specific inhibitor of Golgi mediated secretion in eukaryotic cells (Driouich et al., 1993). It targets a subset of sec7-type GTP exchange factors that catalyse the activation of ARFs which are responsible for formation of COPI-coated and clathrin/AP1 coated vesicles (Nebenfuhr et al., 2002; Zeghouf et al., 2005). BFDA blocks transport of secretory proteins from endoplasmic reticulum to Golgi but most importantly it causes rapid disorganization of Golgi structure, disassembly of cisternal stacks, redistribution of Golgi proteins back to endoplasmic reticulum and possibly inhibits fusion of ER-derived transport vesicles with Golgi (Driouich et al., 1993, Nebenfuhr and Staehelin, 2001; Nebenfuhr et al., 2002). Changes caused by BFDA are reversible (Driouich et al., 1993).

Brefeldin A was shown to inhibit secretion of proteins in both non-encysting and encysting trophozoites of *Giardia* (Lujan et al., 1995). In plants it causes decreased secretion of CW proteins and hemicellulose, inhibition of the transport of polysaccharides and cellulose synthase complexes to the cell surface, inhibition of the synthesis of N-linked glycans and glycosylation of proteins (Driouich et al., 1993; Leucci et al., 2006).

Concanamycin A (ConA) is a specific inhibitor of vacuolar type H^+-ATPases which are maintaining accurate pH gradient along the secretory route. In *Dictyostelium* cells treated with Concanamycin A, endocystosis, exocytosis and phagocytosis are severely impaired. Number of small vesicles decreases significantly and these are replaced by 1-2 large vesicles derived from endo-lysosomal compartment. Moreover, treatment with ConA affects the function of contractile vacuole in this organism, as the treated cells are unable to survive the hypoosmotic environment and eventually lyse (Temesvari et al., 1996).

1.5 Synthesis of cell wall polysaccharides

The key enzymes involved in the synthesis of CW glycans (cellulose, pectins, hemicelluloses and protein-linked glycans) are glycosyltransferases (GT). With the exception of cellulose these glycans are synthesized in Golgi apparatus (Buckeridge et al., 2000; Oikawa et al., 2013). Apart from glycosyltransferases, other transferases, nucleotide sugar interconverting enzymes and nucleotide sugar transporters are necessary for the polysaccharides synthesis. Many of the plant CW polysaccharides are further modified through methylation, acetylation or ferulylation (Oikawa et al., 2013).

1.5.1 Cellulose synthesis

Cellulose, a polysaccharide consisting of glucose residues connected by β-1,4-linkages is a major polysaccharide present in many plants, algae, animals (Urochordates), Stramenopiles, in the cyst wall of *Acanthamoeba* spp, *Hartmanella glabea*, *Dictyostelium discoideum*, *Naegleria gruberi* and many more (Metcalf et al., 2003; Nobles and Brown, 2007). The presence of cellulose in the surfaces or walls of so many different eukaryotic organisms is not surprising: it is relatively cheap to manufacture, difficult to digest and extraordinary strong (Niklas, 2004).

Cellulose in nature never occurs as a single chain but exists as a composite of many chains also known as microfibrils where single chains associate by hydrogen bonding and van der

Waals interactions (Delmer and Amor, 1995). Most cellulose is produced as crystalline cellulose or cellulose I consisting of glucan chains parallel to each other, which can be further divided to Iα (algae) and Iβ (cotton, wood, tunicates, ramie) depending on the physical properties affected by crystal packing, molecular conformation and hydrogen bonding(Saxena and Brown, 2005).

One microfibril of cellulose is usually composed of 30-36 β-1,4-linked glucan chains each containing up to 14 000 glucose residues (Delmer and Amor, 1995; Somerville et al., 2004; Somerville, 2006).

Genes encoding cellulose synthases (CESA) without exception seem to display great similarity between prokaryotic and eukaryotic sequences; the latter probably acquiring the gene by multiple lateral gene transfers from bacteria (Nobles and Brown, 2004; 2007).

10 different CESA genes in *Arabidopsis thaliana* were identified so far, out of these minimum three (CESA1, CESA3 and CESA6) participate in the cellulose synthesis during primary CW assembly and another three (CESA4, CESA7 and CESA8) during secondary CW synthesis (Reiter, 2002; Somerville et al., 2004). In contrast to the plants, single gene of cellulose synthase (DcsA) seems to be clearly sufficient for synthesis of this polysaccharide in *Dictyostelium* (Blanton et al., 2000; Doblin et al., 2002).

All CesA proteins are Mg^{2+} dependent enzymes, which contain eight transmembrane domains, D,D,D, QxxRW motif (typical for all β-glycosyltransferases) believed to be part of the active site, a putative zinc-binding domain participating in protein-protein interactions and hypervariable region (Liu and Hassid, 1970; Delmer and Amor, 1995; Doblin et al., 2002; Reiter, 2002; Nobles and Brown, 2007).

In vascular plants cellulose is synthesized by multimeric cellulose synthase complex which contains minimum three types of glycosyltransferases (cellulose synthases) arranged into a hexameric rossette (Taylor et al., 2000; Doblin et al., 2002; Somerville, 2006, Mutwil et al., 2008). Multienzyme terminal complexes with different architecture have been also observed in algae, *Acetobacter xylinum*, urochordates and *D. discoideum* (Nobles and Brown, 2007). These can be organized into either single rows of linear terminal complexes (*A. xylinum*, brown algae and some red algae), multiple rows (glaucophycean algae, red algae, *D. discoideum* and tunicates) or diagonal rows (*Vaucheria hamata*) (Saxena and Brown, 2005).

Each of the six subunits of the plant cellulose complexes contains five or six CesA proteins (altogether 36) assembled into 1 rosette structure, with each protein synthesizing one β-1,4-glycan (Somerville et al., 2004; Somerville, 2006). The synthesis takes place while the

complexes oriented by cortical microtubules are moving along the plasma membrane with average velocity of 330 nm/min which corresponds to adding 300-1000 glucose residues per chain per minute (Paredez et al., 2006).

Cellulose is a linear homopolymer of β-1,4-glucose, where each subunit is rotated 180° with respect to its neighbour to form a flat ribbon. Its synthesis might be viewed as simple polymerization of glucose residues from a substrate such as UDP-glucose which is added to the polymer but with regard to the stereochemistry, the process is much more complicated and so far not clearly understood. To achieve ribbon-like structure of cellulose, it was proposed that CesA proteins work as dimers, with the active sites oriented such that two glucose residues are added in their correct orientation simultaneously (Delmer and Amor, 1995; Doblin et al., 2002; Taylor, 2008).

The role of cellotriosyl sitosterol and other glycosylate sitosterol as intermediates (Delmer, 1999) or primers (Peng et al., 2002) for the cellulose synthesis were proposed, but never satisfactory demonstrated. Whether the process requires a primer or not is not known, however no exogenous primer was required to initiate synthesis of cellulose in vitro (Somerville et al., 2004).

During the synthesis, UDP glucose is thought to bind to active site of the complex on the cytoplasmic face of plasma membrane with the polysaccharide being extruded through the membrane presumably through a pore type structure into the wall (Doblin et al., 2002). The factors influencing the crystallinity of cellulose were not clearly demonstrated so far but it seems that no proteins but rather different distinct polysaccharides which are accompanying cellulose in the CW are implicated in the process (Somerville et al., 2004; Saxena and Brown, 2005; Joshi and Mansfield, 2007, West et al., 2008).

Correct cellulose synthesis requires apart from others also functional endo-1,4-β-glucanase/cellulase, which was proposed to be involved in chain termination or removal of defective complexes from growing microfibrils (Reiter, 2002; Molhoj et al., 2002).

Generally the walls containing cellulose in the non-plant cells are only poorly characterised, but it is believed, that apart from cellulose other polysaccharides are present (West et al., 2008). It is not known so far, whether all organisms use similar pathways to synthesize cellulose or there are alternate pathways used by different organisms (Matthysse et al., 1995).

Also walls of many of these organisms contain proteins with cellulose binding activity which role except for some is still not understood clearly. Many of these proteins are cellulases which might participate in the modification of the cyst/spore wall or have role in the successful digestion of the wall during excystation (Metcalf et al., 2003).

1.5.1.1 Inhibitors of cellulose synthesis

Inhibitors of cellulose synthesis have got different, often unknown mode of action and are used as a tool to clarify the mechanism of cellulose synthesis. However, none of known inhibitors is able to eliminate the synthesis completely (Kiedaisch et al. 2003). Decreased amount of cellulose usually leads to accumulation of ectopic lignins and changes in the composition of matrix polysaccharides (Shedletsky et al., 1990; Manfield et al., 2004; Taylor, 2008).

The most common inhibitors used in different studies and commercionally are 2,6-dichlorobenzonitrile (DCB), N-(3(1-ethyl-1-methylpropyl)-5-isoxazolyl)) (Isoxaben) and 1-cyclohexyl-5-(2,3,4,5,6-pentafluorophenoxy)-1lambda4,2,46-thiatriazin-3-amine (CGA 325'615 or GCA) (Kiedeisch et al., 2003).

Isoxaben seems to directly interact with certain CesA proteins, depleting them from cellular membrane but also seems to prevent characteristic orientation of the microtubules (Fisher And Cyr, 1998; Heim et al., 1989; Peng et al., 2002; Manfield et al., 2004). Its effectiveness depends on particular aminoacids composition on one of several cellulose synthase enzymes (CesA3 and CesA6) that cooperate in the process (Scheible et al., 2001, Desprez et al., 2002; Manfield et al., 2004; Paredez et al., 2006; Brabham and DeBolt, 2013).

CGA 325'625 inhibits synthesis of crystalline cellulose possibly due to interference with dimerization of cellulose synthases and leads to accumulation of noncrystalline cellulose (Doblin et al., 2002; Kiedaisch et al., 2003; Brabham and DeBolt, 2013).

Rosette cellulose synthase complexes deposition into the cell membrane and their subsequent fast depletion are caused by cellulose synthase inhibitor AE F150944. It does not seem to be effective in organisms with other architecture of cellulose synthases complexes (Kiedaisch et al., 2003).

Main effect of dichlorobenzonitrile seems to be either disarrangements (Mizuta and Brown, 1992) or stabilisation of the cellulose synthase complexes subunits in plasma membrane and subsequently preventing them from movement and synthesis process (Doblin et al., 2002; Mutwil et al., 2008). It has got broad range of efficiency regardless of the terminal complexes architecture, but it seems not to be effective in bacteria (Yu and Atalla, 1996; Brabham and DeBolt, 2013).

Calcofluor white, fluorescent brightener detecting polysaccharides with β-1,4-linkages such as cellulose and chitin can be used to inhibit the cellulose synthesis in vitro (Mizuta and

Brown, 1992; Mori and Lorenza, 1996). It was shown to lead to deformation of subunit structure of terminal complex into fragments or aggregates (Mizuta and Brown, 1992).

1.5.2 Lichenin synthesis

(1,3;1,4)-β-D-glucans (MLGns or lichenin) is rather scarce in the nature. So far it was isolated from the walls of grasses (Poaceae) and related families from the Poales, *Equisetum arvense* and some fungi including basidiomycetes and ascomycetes but not from the walls of dicotyledons or most monocotyledonous plants (Sorensen et al., 2008; Doblin et al., 2009). MLG has got a unique structure as it is unbranched and unsubstitued and contains a single type of monomeric unit, with two distinct linkage types that are arranged in nonrepeating, but non-random fashion (Burton et al., 2006). In barley usually two or three adjacent (1,4)-linked beta-D-glucosyl residues are separated by a single (1,3)-linked β-D-glucosyl residue. It could be therefore also viewed as a polymer of cellotriosyl and cellotetraosyl residues linked by single (1,3)-β-linkages (Burton et al., 2008). The ratio of odd-numbered cellodextrin oligomers is generally about two-fold greater than the even number but in different organism, the structure might be different: *Cetraria islandica* (lichen) has a β-glucan with predominately cellotriosyl unit structure comprising 86% of the polymer (Buckeridge et al., 2000); fungal MLGs have different structures as DP3 or DP4 predominates found in Poales (Burton and Fincher, 2012).

CslF and CslH family of genes seem to be essential for synthesis of (1,3;1,4)-β-D-glucans (Burton et al., 2006; Doblin et al., 2009; Taketa et al., 2012) however, it cannot be ruled out that other enzymes, proteins or cofactors participate in the synthesis (Burton et al., 2006).

The actual synthesis of lichenin is not clearly understood so far but there are several theories proposed (Reviewed by Burton and Fincher, 2012). MLGs seem to be synthesized in the endoplasmic reticulum and Golgi associated vesicles as both CslH enzyme and MLGs were detected in these compartments and the synthesis of lichenin was achieved in in-vitro experiments with enriched Golgi membranes (Urbanowicz et al., 2004; Doblin et al., 2009; Carpita and McCann, 2010).

1.5 A model organism: Free-living pathogenic amoebae

Free living pathogenic amoebae are represented by four species: *Naegleria fowleri, Acanthamoeba* spp., *Balamuthia mandrillaris* and *Sappinia dipploidea.* In my study I focused

on two sister organisms *Acanthamoeba* spp. and *B. mandrillaris*, therefore no detailed explanation considering other two amoebae is provided in this thesis.

1.5.1 Bit of history

Acanthamoebae were first discovered as contaminant growing in a yeast culture of *Cryptococcus paraoseus* in 1930 by Sir Aldo Castellanii. Later, Culbertson has proven their pathogenic potential during development of the polio vaccine (Culbertson, 1958). Plaques appeared in cell cultures inoculated with the vaccine as well as the uninoculated control during safety testing. Moreover, cortisone treated monkeys and mice developed prostrating illness followed by death after inoculation of unfiltered, uncentrifuged and undiluted culture fluids. During histological examination of the brain tissue, amoebae were discovered and described as A-1 strain (Culbertson et al. 1958). Culbertson classified the organism into the *Hartmanella-Acanthamoeba* group. However, based on current knowledge, these two represent different genera and all references are recommended to be corrected accordingly (Martínez et al., 1997).

In 1965 first cases of human encephalitis caused by free living amoebae were discovered by Fowler and Carter in Australia (Fowler and Carter, 1965) and the pathogenic agent was described as *Naegleria fowleri* (Carter, 1968). It was demonstrated for the first time that the barrier between free-living and parasitic lifestyle can be overcome in some organisms and lead to fulminant infections in human. First human cases of *Acanthamoeba* encephalitis were reported in the seventies (Kenney, 1971; Jager and Stamm, 1972; Robert and Rorke, 1973); the first infection in AIDS patient was described in 1986 (Gonzalez et al. 1986). *Acanthamoeba* keratitis (AK) was reported for the first time in 1974 in a patient with previous trauma of the eye (Nagington et al., 1974). Since 1985 is the number of keratitis cases gradually increasing each year (Visvesvara et al., 2007) corresponding to the increased usage of contact lenses not only for the medical but also cosmetic purposes but also to the change of the technology of their production (Niederkorn et al., 1999).

Amoebae lately described as *Balamuthia mandrillaris* were first discovered as a causative agents of meningoencephalitis of baboon (*Papio sphinx*) that died in San Diego ZOO in 1986 (Visvesvara et al., 1993). Very soon after, the first human infection was identified in HIV positive intravenous drug user who died very soon after developing symptoms corresponding to multiple granulomatous lesions in the brain (Anzil et al., 1991). Once was *Balamuthia mandrillaris* established in the culture and antibodies against it raised in rabbits, it was possible to examine the old autopsy material from patients dying of unidentified amoebic

meningoencephalitis in the past by immunofluorescence microscopy, revealing that approximately 35 cases were caused by this amoeba. Based on this information, the first „real“ confirmed case occurred in 1974 in a 47 year old female suffering from diabetes mellitus (DM) (Duma et al., 1978, Rowen et al., 1995). The first *Balamuthia* encephalitis case in Europe occurred in a 3 year old child in Czech Republic in 1995 unfortunately the organism was not recovered in a culture (Kodet et al., 1998). The disease seem to be more prevalent in warmer climate zones, especially in South America and warmer temperate zones of the USA (Schuster et al., 2004); the infections in Europe remain rather scarce, so far reported from Czech Republic, Portugal and Great Britain (Jayasekera et al, 2004; Kodet et al., 2005; Tavares et al., 2006).

The interest in the studying of free-living amoebae started in Czech Republic by Dr Lubor Červa after 16 fatalities caused by *Naegleria fowleri* occurred between 1962-1965 and it continued after the first case of *Balamuthia mandrillaris* in Europe was diagnosed in the Laboratory of tropical diseases, 1st Faculty of Medicine, Charles University in Prague, CR in cooperation with Dr Govinda Visvesvara, CDC, USA.

1.5.2 Classification

Acanthamoeba and *Balamuthia* are sister genera of group Amoebozoa, sub-group Acanthamoebidae (Adl et al., 2005). The sequence similarity between *B. mandrillaris* and *Acanthamoeba* is between 17.9-21.1% (Booton et al., 2003a, b). Sequence variation in the 16S rRNA genes of all *Balamuthia* isolates tested to date range from 0 to 1.8% therefore all have been placed in a single species *Balamuthia mandrillaris* (Booton et al., 2003a, b).

Acanthamoebae were previously classified into 3 different groups based on the cyst morphology and size (Pussard and Pons, 1977; reviewed in Schuster and Visvesvara. 2004; Khan, 2006):

- Group I (*A. astronyxis, A. comandoni, A. echinulata, A. tubiashi*): amoebae with large cysts (18 μm or more) with stellate endocyst and smooth or wrinkled ectocysts..
- Group II (*A. mauritaniensis, A. castellanii, A. polyphaga, A. quina, A. divionensis, A. triangularis, A. lugdunensis, A. griffini, A. rhysodes, A. paradivionensis, A. hatchetti*): amoebae with cysts smaller than 18 μm with polyhedric, globular, ovoid or stellate endocysts and wavy ectocyst.

- Group III (*A. palestinensis, A. culbertsoni, A. royreba, A. lenticulata, A. pustulosa*): amoebae with cysts larger than 19 μm with globular or ovoid endocysts and smooth or wavy ectocysts..

However, as the morphology of the cyst depends on several different factors such as a ionic strength of the growth medium or used encystation medium and acanthamoebae might even lose their ability to encyst after prolonged axenic cultivation, this grouping is no longer used (Sawyer, 1971; Köhsler et al., 2009).

Current classification is based on sequencing of the 18S rRNA genes. 17 genotypes of *Acanthamoeba* were so far described (T1-T17) with each genotype exhibiting 5% or more sequence divergence from the others (Stothard et al., 1998, Gast 2001, Marciano-Cabral and Cabral, 2003, Schuster and Visvesvara, 2004; Khan, 2006, Köhsler et al., 2006; Magliano et al., 2012).

ECOLOGY

Acanthamoeba spp. and *B. mandrillaris* belong to free-living aerobic protists occurring worldwide. Because of their capability of infecting mammals and cause a disease, they are also known as amphizoic amoebae (Schuster and Visvesvara, 2004).

There are several species of acathamoebae which were shown to be pathogenic (*A. castellanii, A. culbertsoni, A. hatchetii, A. healyi, A. polyphaga, A. rhysodes, A. astronyxis*, and *A. divionensis*). *B. mandrillaris* is believed to exist as a single species of the genus *Balamuthia* (Booton et al., 2003a,b).

Acanthamoebae were isolated from a variety of environments: water including the seawater, dust, soil, air conditioning units, ventilation ducts, pipe lines, cooling towers of electric and nuclear power plants, toxic waste dumpsites with high levels of pesticides, herbicides, pharmaceuticals, and heavy metals, mouth and different body materials from immunocompetent individuals (Visvesvara et al., 2007; Johnston et al., 2009). These organisms feed by phagocytosis preferring gram-negative bacteria (Bottone et al., 1994; Khan, 2006). While feeding, amoebastome-like surface structures that ingest epithelial cells were observed on the surface of acanthamoeba cells (Omana-Molina et al., 2004). Acanthamoebae tolerate wide range of osmolarity, enabling them to survive in distilled water, tissue culture media or sea water whereas, *B. mandrillaris* is very sensitive to the changes in osmolarity and does not readily tolerate either hypo- or hyperosmotic conditions (Schuster, 2002). Only three successful isolations of *B. mandrillaris* from the environment were reported so far. In two cases the organism was isolated from unrelated soil samples in California

(Schuster et al., 2003; Dunnebacke et al., 2004), the last isolate was derived from dust in a public building in Tehran (Niyyati et al., 2009). Difficulties in the isolation were explained by several factors such as slow growth, restriction to certain environmental niches, contamination and subsequent overgrowth of the sample by other organisms (Visvesvara et al., 2007; Matin et al., 2008). Moreover, it seems rather likely, that *Balamuthia* prefers to reside in a nutrient rich environment (Schuster and Visvesvara, 2007). In vitro, it does not feed on bacteria, but prefers trophozoites, but not cysts of other free-living amoebae and might do so in the nature (Visvesvara et al., 2007; Matin et al., 2006a). It appears that balamuthias have got rather intriguing mechanism of feeding in the tissue culture by direct invasion of the pseudopodia and/or whole balamuthias into the interior of the tissue cell and subsequent feeding on cytoplasm and nuclei (Dunnebacke, 2007).

1.5.3 Life cycle

Both, *Acanthamoeba* spp. and *B. mandrillaris* have two stages in their life cycles: a vegetative amoebic trophozoite and a dormant cyst. Encystation is a protective response of the organisms to the unfavourable conditions of the environment (Weisman, 1974; Matin et al., 2008a), whereas upon appropriate conditions amoebae are activated, reverted to the trophic form and start to feed and multiply (Chavez-Munguia et al., 2007).

1.5.3.1 Trophozoite

The trophozoite of *Acanthamoeba* measures 15-50 μm. It grows and multiplies in 25°C with generation time 8-24 hours, although many isolates are able to grow in higher temperatures. In the outer environment, acanthamoebae feed on bacteria, algae, yeast or small organic particles (Khan, 2006).

Trophozoite cell possesses a single nucleus with a large nucleolus, mitochondria, well-developed endomembrane system composed of Golgi complex and endoplasmic reticulum, many digestive vacuoles, lipid droplets and glycogen particles. There are individual microtubules in the cytoplasm but no centriole has been identified (Bowers and Korn, 1968; Lasman 1982; Martínez and Visvesvara, 1997, Gonzales-Robles et al., 2001; Khan, 2001; Khan, 2006). Typical features of the *Acanthamoeba* trophozoite are spiny like protrusions known as acanthopodia and a large contractile vacuole. Acanthopodia which are constantly extending and retracting from the cell surface participate in cell adhesion, movement and capture of prey. It seems that the pathogenic isolates exhibit higher number of acanthopodia than non-pathogenic ones (Bowers and Korn, 1968; Lasman 1982; Martínez and Visvesvara,

1997, Gonzales-Robles et al., 2001; Khan, 2001; Khan, 2006). Contractile vacuole accompanied by tubular system (spongiome) enables the trophozoite adaptation to habitats with different osmolarity. The vacuole discharges periodically: it fragments into a number of collapsed vesicles which subsequently refill (Bowers and Korn, 1968; Lasman, 1982; Gonzales-Robles et al., 2001).

The trophozoite of *B. mandrillaris* measures from 12 to 60 μm. It is uninucleate with large centrally placed nucleolus but two or three nucleolar bodies can be also seen (Visvesvara et al., 2007). Mitochondria and cisternae of rough endoplasmic reticulum are present in the cytoplasm. The organism reproduces asexually by binary fission (Matin et al., 2008).

During axenic cultivation *Balamuthia* trophozoites are highly polymorphic, ranging from nearly spherical to various amoeboid forms with intense surface activity (Matin et al., 2008). *B. mandrillaris* grows axenically only in a nutrient rich medium (BM-3) or on feeder layers of Vero kidney cells. Generation time of various isolates in BM-3 medium is 20-28 hours (Schuster, 2002).

1.5.3.2 Cyst

Mature cyst is highly resistant, dormant stage which maintains virulence of organism for long period of time (Weisman, 1974). Cysts of acanthamoebae measure between 15-20 μm in diameter and are double-walled. Outer layer of the wall (=ectocyst or exocyst) is wrinkled, the inner wall (endocyst) is usually stellate, polygonal, round or oval (Martínez and Visvesvara, 1997).

Pores or ostioles that are covered by convex-concave plugs referred to as opercula are present at the junction of the ecto and endocyst. These are probably used to monitor the environmental changes (Khan, 2006). Cysts are uninuclear (Martínez and Visvesvara, 1997). Golgi is distributed as small aggregates, contractile vacuole is absent. In the cytoplasm, mitochondriae, lipid droplets, rough endoplasmic reticulum and autolysosomes containing debris of mitochondria, lipid droplets and glycogen particles are found (Bowers and Korn, 1969). Large aggregates of rod-shape elements similar to chromatoid bodies were also described in acanthamoeba mature cysts (Chavez-Munguia et al., 2013).

The uninuclear cyst of *Balamuthia* has triple-layered wall: thin irregular ectocyst, thick electron dense endocyst and middle amorphous fibrillar layer (mesocyst) all lacking pores. They measure from 12-30 μm (Schuster et al., 2003; Visvesvara et al., 2007).

1.5.4 Diseases caused by *Acanthamoeba* and *Balamuthia*

So far, there were more than 200 of cases of *Acanthamoeba* encephalitis and more than 100 *Balamuthia* encephalitis cases described in the literature. The actual number might be much higher as diseases caused by free-living amoebae are diagnosed mostly post-mortem, the diagnosis requires expertise in the field, and cases from Sub Saharan Africa and Asia, countries with predominance of HIV/AIDS are not reported. The actual incidence of the diseases is not really known (Visvesvara et al., 2007) although approximate rate of deaths to the GAE in the HIV/AIDS patients in USA is approximately 1.57 per 10 000 (Khan, 2006).

Amoebic keratitis, infection of the cornea caused by *Acanthamoeba* spp. is a disease mostly affecting immunocompetent contact lens wearers, rarely found in individuals with trauma of the eye. The annual incidence per 10 000 contact lens wearers per year was established as 0.33 in Hong Kong, 0.05 in Holland, 0.01 in USA (Stehr-Green et al, 1989), 0.19 in England (Radford et al., 2002) and 1.49 in Scotland (Seal et al., 1999; Lam et al., 2002, reviewed in Khan, 2009).

1.5.4.1 Amoebic encephalitis

Both, *Balamuthia* and *Acanthamoeba* produce chronic, slowly progressing focal disease of brain called granulomatous amoebic encephalitis (GAE) (Martínez, 1980). Infections of other organs such as skin, lungs or disseminated infections are also reported (Visvesvara et al., 2007, Galarza et al., 2009).

Widely distributed acanthamoebae seem to get in contact with human host quite often as suggested by the prevalence of antibodies in healthy individuals ranging from 3% up to 100% in different studies (Červa, 1989; Curson at al 1980, Chappell et al., 2001) but only in some circumstances are capable of causing a disease. The most common genotype associated with the disease but also mostly prevalent in the nature is T4 genotype (Booton et al., 2005; Maciver et al, 2012) which also seems to be more resistant to the chemotherapy and shows greater virulence (Maghsood et al., 2005).

Acanthamoeba GAE occurs mostly in immunodeficient patients in conditions such as HIV/AIDS, chronic alcoholism, and systemic lupus erythematosus or during bone marrow suppression due to the chemotherapy ((Marciano-Cabral et al., 2000; Marciano-Cabral and Cabral,, 2003). However, altogether 10 cases in immunocompetent previously healthy individuals were also reported (Lackner, et al., 2009; reviewed in Reddy et al., 2011).

GAE caused by *Balamuthia* (BAE) occurs in immunocompromised as well as immunocompetent individuals such as young children or older people (Visvesvara et al.,

1993; Visvesvara et al., 2007). California encephalitis project identified 10 cases of BAE among 3500 cases of meningoencephalitis between 1999 and 2008 (Schuster et al., 2009). The authors identified predisposing factors of the infection such as old or young age, chronic health problems, malnutrition, contact with soil or water (gardening, playing in the dirt, contamination of open wound by soil), prior treatment with corticosteroids and Hispanic ethnicity (Visvesvara et al., 2007; Schuster et al., 2009).

The port of the entry of the infection is thought to be lungs or skin and the amoebae of both genera spread to the CNS hematogenously (Pritzker et al., 2004; Galarza et al., 2009). Intranasal infection with *B. mandrillaris* in mice was shown experimentally (Kiderlen et al. 2004; Kiderlen et al., 2007).

Both, trophozoites and cysts are found in the necrotic tissue of brain (Schuster and Visvesvara, 1994).

It is believed, that pathogenicity of both amoebae is quite similar: release of different proteases, phospholipases, lipases, production of surface like projections resembling the food cups (Alsam et al., 2005; Matin et al., 2006; Matin et al., 2008). While acanthamoebae use mannose-binding protein to attach to the host cells, *B. mandrillaris* was shown to bind to human brain microvascular endothelial cells (HBMEC) by galactose-binding protein (Niederkorn et al., 1999; Matin et al., 2008).

1.5.4.2 *Acanthamoeba* keratitis

Acanthamoeba keratitis (AK) is a vision threatening infection of cornea. It presents with severe pain of the affected eye, blurred vision, congestion of conjunctiva and photophobia, and might lead despite the treatment to decrease of the visual acuity, blindness and enucleation of the eye. Typically, only one eye is affected (Visvesvara et al., 2007).

First cases of AK were considered as rare infections in people with trauma of the eye; however, in the recent years the majority of cases occur in contact lens wearers (Niederkorn et al., 1999).

As approximately 2-10% of the contact lens cases of asymptomatic individuals are contaminated by acanthamoebae (Gray et al., 1995; Niederkorn et al., 1999), it seems that for successful infection, several factors (internal and external) must be present:

a) Corneal abrasion: It is evident that acanthamoebae can invade only a cornea previously damaged: by only a minor often unnoticed abrasion occurring while applying the contact lenses or major trauma of the eye which leads to the seek of the medical care. Moreover, animal models of AK also require corneal abrasion or delivery of trophozoites

directly into the stroma (Niederkorn et al., 1999). On the abrased cornea, the expression of acanthamoeba binding mannose-glycoproteins is 1.8 times higher than on the healthy cornea allowing more trophozoites to attach to the surface and release proteases and other factors contributing to the disease (Alizadeh et al. 1994; Jaison et al, 1998; Clarke and Niederkorn, 2006).

b) Corneal immunity: Levels of secretory IgA (sIgA), protective antibody, was shown to be decreased in the individuals with AK (Leher et al., 1999; Alizadeh et al., 2001; Walochnik et al., 2001; Garate et al., 2006a, b).

c) Low level of hygiene during the cleaning of the contact lenses: Majority of the cases occur in individuals using the tap water in preparation of contact lens solutions or failing to keep the contact lens cases clean, wearing the lenses longer than instructed by manufacturer or wearing them while swimming. Moreover, acanthamoebae are able to adhere more firmly to the used contact lenses than to the new ones (Beattie et al., 2003).

d) Contact lens solutions: Usage of certain contact lens solutions for cleaning represents another risk factor as different contact lens solutions might fail in killing the trophozoites or cysts of acanthamoebae (Johnston et al., 2009).

Once the trophozoites attach, release of proteases, apoptosis of the host cells and other factors contribute to spreading of the amoebas and severity of the disease (Alizadeh et al., 1994; Clarke and Niederkorn, 2006).

1.5.5 Difficulties in the treatment

The treatment of all diseases caused by free-living amoebae is complicated, prolonged and requires the use of combination of drugs. Despite the therapy, the CNS infections are usually fatal (Schuster and Visvesvara, 2004). Similarly, in patients with AK, penetrating keratoplasty or corneal grafting might be necessary to eliminate the organism; moreover, some patients have to undergo enucleation of the eye as a symptomatic relief. In AK cases which are diagnosed early and properly treated approximately 90% of the patients retain visual acuity of 6/12 or better and less than 2% of the patients become blind (Dart et al., 2009). Apart from the pathogenicity of the amoeba, several factors contribute to the unfavourable outcomes: a) no drugs targeting both, trophozoites and cysts exist, the latter representing a highly resistant stage contributing to the possible relapse of the disease b) the multidrug toxicity of the treatment c) level of immunodeficiency in GAE (severe imunodeficiency: HIV/AIDS versus

mild immunodeficiency: DM, alcoholism) (Martínez and Visvesvara, 1997; Dart et al., 2009). Successfully treated individuals may still develop disabilities such as loss of the hearing (GAE) or vision impairment (AK) (Khan, 2006).

Differentiation into dormant, highly resistant cyst represents a major problem for the therapy of the diseases as it was shown in-vitro that acanthamoebae encyst as a response to the presence of resting macrophages or to the therapeutic agents (Marciano-Cabral and Toney, 1998; Chomicz et al., 2005). Cysts created within tissues seem to be the source of the infection in the relapse of the disease as the walls enclosing the organism represent a firm barrier which prevents the uptake of the drug (Turner et al., 2004).

1.5.6 Encystation

Most of the studies on encystation of amphizoic amoebae were done with a genus *Acanthamoeba*; only little information is available about the encystation of *B. mandrillaris* mainly due to the fact that no effective method of in vitro encystation induction was described so far (Schuster and Visvesvara, 1996). *Balamuthia* encysts spontaneously after the consumption of a feeder layer of mammalian cells during co-cultivation, but the encystment is not synchronised and does not allow studying the process closely. In BM-3 medium even after months of axenic cultivation, only a minority of balamuthias spontaneously encyst (Schuster and Visvesvara, 1996). Recently, Siddiqui et al. (2010) showed that addition of galactose but no other sugars enhanced encystation in vitro by up to 70% (Siddiqui et al. 2010). However no other studies are available evaluating the efficacy of this encystment medium and no comparison of the spontaneously formed and induced cyst is available.

Generally, the encystation in amoebae can be divided into three phases: induction, wall-synthesis, dormancy (Weisman, 1976).

1. Induction of encystation

Encystation is a reaction to harsh environmental conditions: increased osmolarity, extremes in temperature and pH, and starvation, especially glucose starvation (Weisman, 1976; Byers et al., 1980; Cordingley et al., 1996; Turner et al., 1997; Dudley et al., 2005). It can be achieved in-vitro by cultivating acanthamoebae on the non-nutrient agar covered with heat-inactivated bacteria where amoebae upon their consumption form cysts or by exposure of the organism to different ‘encystation’ media (Bowers and Korn, 1969; Weisman, 1976; Schuster, 2002). To

complete the process, Ca^{2+} or Mg^{2+} ions are required (possibly as cofactors of cellulose synthase), the role of oxygen remains under discussion (Weisman, 1976; Turner et al., 1997). Encystation medium of Neff and Neff (EM Neff) (1969) resembles starvation condition, Dudley et al (2005) and Cordingley et al. (1996) operate with high osmolarities, Chagla and Griffiths (1974) use 50 mM Mg^{2+}. In the study of Yang and Villemez (1994) the encystation was initiated upon binding of monoclonal antibodies to 40kDA receptor found on surface of trophozoites. Later it was hypothesized, that this receptor might participate in the detection of high osmolarity (Cordingley et al., 1996).

Encystation is a response to chronic, prolonged exposure to the harmful conditions or substances as amoebae which are transferred into the growth medium within the first hours after induction usually do not differentiate but continue to feed and multiply. However, once the signals remain trophozoites will either differentiate into mature cysts or lyse (Weisman, 1976).

2. Wall synthesis

The first detailed information about encystation observing the CW synthesis, assembly and composition comes from the ultrastructural study of encystation done by Bowers and Korn in 1969 which is summarized together with results obtained by other authors (Weisman, 1976; Lasman, 1982; Chavez-Munguia et al., 2013) in the following text.

Four morphological stages were identified by electron microscopy during encystation: trophozoite, precyst, immature cyst and mature cyst.

The cells remain ameboid in the encystation medium within 6-7 hours after induction of encystation but some changes already take place: endoplasmic reticulum is reorganised, Golgi increases in size and many small vesicles appear to bud off its cisternae, continuing to the cell surface where they release their content. *Acanthamoeba* degradates unnecessary particles and cellular materials in autolysosomes which volume significantly increases in size. Majority of autolysosomes are later expelled from the cytoplasm and are found in surrounding medium or within the exocyst wall. The nuclear and nucleolar volume decreases in size, the levels of RNA, proteins, triacylglycerides and glycogen gradually declines. Water expulsion vesicle collapses. Coiled lamellate structures appear within the elongated mitochondria very soon after induction of encystation (1-2 hours), but disappear during the later stages most probably as reaction to the starvation than the encystation itself.

Later (12-14 hours after induction), acanthopodia retract and can be detected only as small protuberances, the cell becomes rounded with dense cytoplasm most probably due to the dehydration. Mitochondria decrease in size and numbers while the amount and concentration of lipid droplets rises dramatically.

Exocyst is firstly observed as a discontinuous layer of amorphous material 200 nm thick which appears before the cell rounds up. By the time the cell is completely rounded this layer is covering the entire surface. Structures resembling ostioles but not clearly distinguished are also present on the surface of the immature cyst.

3. Dormancy

In mature cyst, both layers of the CW are already present. Tridimensional visualization of ultrastructural organisation of the *A. polyphaga* CW (Lembruger et al., 2010) showed that the exocyst is an irregular and compact layer about twice as thick as the endocyst covering the whole surface of the cell including the operculum (Chavez-Munguia et al., 2013). Underneath, in between exo- and endocyst, there is a clear electron-lucent, amorphous zone of average thickness of 840 nm where the cytoplasmic and membranous debris are often found. The endocyst composed of 10 nm thick fibrous structures has a biphasic organisation: the fibres resembling plant cellulose are compact close to the cell surface becoming much looser in the outer region. There are multiple sites of exo- and endocyst fusion forming a bow shape space referred to as ostiole. Ostiole does not contain exocyst or endocyst material and is occupied by large bell shaped structure also known as operculum, which lies close to the underlying plasma membrane (Bowers and Korn, 1969, Chavez-Munguia et al., 2013). The median cell volume of a mature cyst is decreased for about 80% and cell surface for more than 65% when compared to that of a trophozoite (Bowers and Korn, 1969).

1.5.7 Composition of the cyst wall layers

Acanthamoeba CW is composed of 33% of protein, 4-6% lipid, 35% carbohydrates , 8% ash and 20% unidentified materials (Neff and Neff, 1969).

It seems that the exocyst is mainly composed of hydroxyproline rich acid-resistant proteins but lipids and cellulose were also found in this layer (Bowers and Korn, 1969; Neff and Neff, 1969; Barrett and Alexander, 1977). The endocyst is thought to be composed mainly of

cellulose, although the presence of other polysaccharides, proteins or lipids cannot be ruled out. Cellulose represents approximately 10% of the total dry weight of the cyst, but is thought to represent approximately 1/3 of the endocyst (Tomlison and Jones, 1962).

Among proteins, nine minor peptides, 70 kDA protein and 21kDA cyst specific protein were so far detected in the CW, but their exact location and function remains unknown (Rubin et al., 1976; Neff and Neff, 1969, Hirukawa et al., 1998). In previous studies aimed at the carbohydrate composition of the CW of *Acanthamoeba* it was found out that apart from the glucose, also galactosamine (3%), mannose and glucosamine residues are present (Stewart and Weisman, 1974, Elloway et al., 2004). More detailed results come from the latest study by Dudley et al. (2009) who has shown that carbohydrates represent 0.7% of the CWs material. Among these, majority is represented by galactose (48.1%) and glucose (44.4%), followed by mannose and xylose (3.7%). Intact cysts contains 9.1% carbohydrate; majority most probably in the form of glycogen. According to the glycosyl linkage analysis 4-linked glucosylpyranose represents 22% and 3-linked galactopyranose represents 28.6% of the glycosyl residues. Other detected linkages were these: 3,4-linked galactopyranose (13.6%), 4,6-linked mannopyranose (7.8%), 3,6-linked galactopyranose (7.2%), 5-linked xylofuranose (7%), 3,4-linked glucopyranose (6%), 2,4-linked gluco or galactopyranose (4.4%) and terminal mannopyranose (3.2%) (Dudley et al., 2009).

Different reported amounts of the CW carbohydrates in the above mentioned studies could be due to the fact that the composition of the CW might differ among genotypes and could be influenced by axenic cultivation methods and method used to obtain the CWs (Stratford and Griffiths, 1978; Dudley et al., 2009).

Only little is known about the composition of the triple-layered CW of *Balamuthia mandrillaris* and the current knowledge is based on two studies (Siddiqui et al., 2009a, b). The presence of polysaccharides with β-1,4-linkages in the CW of *Balamuthia* cysts was suggested due to the stainability of the walls by Calcofluor white and PAS (periodic acid Schiff) and the observed inhibition of encystation by cellulose synthesis inhibitors Calcofluor white (0.05%) or Dichlorobenzonitrile (10 μM). The carbohydrate analysis of the CW of *B. mandrillaris* gave surprising results: in intact cysts only 0.1% carbohydrates represented by 71.5mol% of mannose and 28.5mol% of glucose were detected. In purified CWs 0.3% carbohydrates were represented by 20.9mol% of mannose, 79.1mol% of glucose and trace amounts of galactose. The linkage analysis showed CW carbohydrates with both linear and branching saccharides with a relatively large amount of terminal mannopyranose (29.3%) and a lesser amount of terminal galactopyranose (5.9%) moieties as well as 1→3-linked

galactopyranose (15.6 %), 1→5 linked xylofuranose/4 xylopentofuranose (13.3 %), and 1→4-linked glucopyranose (23.0%). .

1.5.8 Molecular and biochemical basis of encystation

Molecular mechanism of encystation in acanthamoebae were so far only partially described although in the recent years more studies aimed at the process were published. However, similarly to other protozoans, the process of encystation is still far from understood (Fouque et al., 2012).

Recently the results of the *A. castellanii* Neff strain genome sequencing were published so it could be expected that the number of publications focusing on molecular basis of encystation will increase (Clarke et al., 2013).

Differentiation into the mature cyst in acanthamoebae redirects the metabolism of the cell to the synthesis of CW, decrease in the cytoplasmic mass and dehydration (Bowers and Korn, 1969, Orfeo et al., 1999). It seems that the cellular components are degraded to provide the material for the synthesis, energy and removal of the excess of the unnecessary material from the amoebae before the cyst formation is completed (Lasman, 1982).

Several different genes participating in the encystation (3 cellulose synthases, cellulase, cellobiosidase, trehalose-6-phosphate synthase) have been identified in the genome of *Acanthamoeba* but their role in differentiation was studied only in few cases (Aqeel et al., 2012)**.**

According to the latest results it seems that encystation is associated with the upregulation of genes that encode proteins with homology to xylose isomerase and Na P-type ATPase but also subtilisin-like serine protease, cysteine proteases, CSP21, protein kinase C, heat shock protein, culling 4, autophage protein 8, ubiquitin-conjugating enzymes and enolase (possibly participating in glycolysis regulation) (Moon et al., 2007; Moon et al., 2008a,b; Moon et al., 2009; Bouyer et al., 2009; Leitsch et al., 2010; Fouque et al, 2012).

Despite the fact that serine and cystein proteases are essential for encystation and excystation, their exact role is so far not understood (Dudley et al., 2008; Moon et al., 2007; 2008b; Leitsch et al., 2010). While it seems that cysteine proteases play only a discrete role during encystation and they do not seem to participate in degradation of cellular proteins (Leitsch et al., 2010), serine proteases seem to be essential for degradation of macromolecules in the autophages (Moon et al., 2008).

Cyst specific protein of 21kDa (proposed encystation marker) is specifically expressed during early stages of encystation (12 hours post induction) similarly to the acid-resistant proteins (8 hours post induction) (Hirukawa et al., 1998; Lloyd et al., 2001; Chavez-Munguia et al., 2013) but while acid-resistant proteins seem to be mainly structural part of the exocyst, the role of CSP21 or its exact localisation was so far not clearly demonstrated.

Cellulose is CW polysaccharide synthesized de-novo during encystation by cellulose synthases (Potter and Weisman, 1972). Their activity increases significantly after 12th hour post induction and continues approximately up to 30 hours post induction (Potter and Weisman, 1971) corresponding to the endocyst formation. Cellulose synthesis seem to be of crucial importance for correct encystation as its silencing by specific siRNAs (Aqeel et al., 2012) or inhibition by dichlorobenzonitrile (DCB) in concentrations from 240 to 480 μM inhibits the encystation process (Dudley et al., 2007), however it does not seem to abolish the staining of the organism by Calcofluor white detecting polysaccharides with β-1,4-linkages such as cellulose or chitin.

Correct cellulose synthesis apart from others requires sufficient amount of substrate namely UDP-glucose, and lipids and phospholipids as well as glycogen were proposed by previous studies as a source of this carbohydrate during the encystation (Mehdi and Garg, 1987; Stewart and Weisman, 1974). During the cyst formation the activity of isocitrate dehydrogenase decreases and there is increase in the activity of isocitrate lyase suggesting the shift from the tricarboxylic acid cycle (TCA) to glyoxylate pathway. This pathway centers on the conversion of acetyl-CoA to succinate for the further synthesis of carbohydrates (Mehdi and Garg, 1987) which could be used as a source of „brick material“ for cellulose synthesis. However, in the study of Stewart and Weisman (1974) this hypothesis was not confirmed. Glycogen, another possible source of glucose, can be degraded by enzymatic cleavage via glycogen phosphorylase or by hydrolytic degradation (Jang and Gomer, 2005; Siddiqui et al., 2011). In study of Weisman et al. (1970) it was shown that observed decrease in glycogen was due to its degradation rather than decrease in its synthesis (Weisman, 1976). Moreover, the level of cAMP rises significantly during first hours of encystation, possibly participating in the conversion of glycogen phosphorylase to its active form by phosphorylation of serine residue as in other eukaryotic cells (Jang and Gomer, 2005; Siddiqui et al., 2011).

Trehalose synthesized by trehalose-6-phosphate synthase was proposed to be involved in protection from desiccation and stress adaptation of cysts, but so far not studied further (Anderson et al., 2005).

There are no detailed information about encystation of *B. mandrillaris* but is seems that the process requires de novo RNA and protein synthesis and can be inhibited by cycloheximide, flucytosine, artemisine and cytochalasin D (Siddiqui et al., 2007; Siddiqui et al, 2010).

1.5.9 Resistance of cysts

Ability of acanthamoebae to encyst is crucial for their wide distribution in the environment, the survival and the resistance to the available therapy (Lemgruber et al., 2010).

Cyst of *Acanthamoeba* spp. is not destroyed by common free chlorine concentration of 4 μg/ml and is still viable upon exposure 3 hours to 40 μg/ml of free chlorine (de Jonckheere and van Van de Voorde, 1976). It is able to withstand prolonged starvation, cold, freezing, heat (60°C exposure for 30 minutes), extremes in the pH, osmolarity, radiation from the Cobalt-60 source, UV irradiation of 800 mJ/cm2 and desiccation for more than 20 years (Bowers and Korn, 1969; Weisman, 1976; Stratford and Griffiths, 1978; Aksozek et al., 2002; Sriram et al., 2008). Moreover, when stored in 4°C it can remain viable for 25 years, while the organism maintains its pathogenicity (Mazur et al., 1995). It was observed that cysts can be formed as a response to the treatment (Chomicz et al. 2005) or as a result of the presence of resting macrophages as shown by in-vitro experiments (Marciano-Cabral and Toney, 1998). The gradual increase of resistance takes part between 12th and 24th hour after induction of encystation corresponding to gradual decrease of sorption of biocides and period when the cellulose is synthesized (Turner et al., 2004).

Most of drugs used in the chemotherapy of *Acanthamoeba* infections therapy act mainly on trophozoites (Khan, 2006): inhibit DNA synthesis (propamidine isethionate) or are membrane-acting cationic biocides (polyhexamethylene biguanide, chlorhexidine digluconate). Both, polyhexamethylene biguanide (PHMB) and chlorhexidine are cysticidal only when used in high concentrations, 200-500 μg/ml and 100 μg/ml, respectively (Khunkitti et al., 1998; Kong and Chung, 1998; Turner et al., 2004). Moreover, cysts have been shown to be resistant in-vitro to imidazoles, caspofungin, voriconazole and miltefosine, to 0.1% propamidine isethionate, 1% neomycin or 1% miconazole (Saunders et al., 1992, Dart et al., 2009).

The CW seems to represent effective barrier stopping the uptake of the drug to the organism. Presence of cysts and their formation within infected tissue therefore represents a significant problem in the chemotherapy (Marciano-Cabral and Cabral, 2003; Niederkorn et al. 1999):

their recrudescence might be the reason for prolonged severe keratitis in patients not responding to the treatment or high fatalities of the CNS infections (Niederkorn et al., 1999).
The cyst of *Balamuthia* was shown to be resistant to high temperatures up to 70°C for 60 minutes, freezing-thawing procedures (5x), UV light exposure up to 60 minutes (200 mJ UV cm -2), chloride exposure up to 25 p.p.m chlorine, pentamidine isethionate, and SDS treatment (Siddiqui et al., 2008).

1.5.10 Contact lenses and *Acanthamoeba* keratitis cases

AK is a disease affecting mostly immunocompetent contact lens wearers. As mentioned previously, approximately 2-10% of asymptomatic wearers of contact lenses have their lens cases contaminated by acanthamoebae (Niederkorn et al., 1999). Considering 6.1 billion contact lens wearers in the world in 2010 (Nicholas, 2011), up to 600 million people might be at potential risk of developing AK.
Between 2004 and 2007 the number of AK cases increased dramatically in USA. They were connected to the usage of Advanced Medical Optics (AMO) Complete MoisturePlus multipurpose contact lens solution, which was subsequently recalled off the market (Johnston et al., 2009). The amoebicidal and cysticidal effect of contact lens solution is therefore of crucial importance in the prevention of the disease. Unfortunately, when 11 different contact lens solutions were evaluated for their efficacy, only one had significant effect in inactivation of cysts (Johnston et al., 2009). In the study of Boost et al. (2012), none of the three tested multipurpose contact lens solutions achieved a 1-log reduction in viability of three *Acanthamoeba* species within the manufacturer's recommended disinfection times.
Currently there are no official guidelines for testing the efficacy of the contact lens solutions against *Acanthamoeba* spp. Without an accepted standard, the procedures used and reported in studies that test contact lens solutions are highly variable (Johnston et al., 2009). As a result, different reports are available addressing the survival of acanthamoebae after exposure to various agents: Beattie et al. (2002) showed that acanthamoebae are sensitive to hydrogen peroxide system used in contact lenses when exposed for more than 6 hours. However, multipurpose contact lens solution containing hydrogen peroxide in the study of Johnston et al. (2009) did not have the same effect.
Due to the different results by different authors, it was suggested that strain age, number of passages in axenic culture and also method of encystment have to be taken into consideration

when evaluating the resistance of the cysts (Hughes et al., 2003). However, the effect of additives and the response of the organism to the solution have also to be taken into consideration.

LIST OF ORIGINAL PAPERS

Results of this thesis have been published in 3 original articles which can be found in Appendix. Two articles are in preparation for publication. Yet unpublished results are also included to chapter summarizing the thesis results.

Glycogen phosphorylase in Acanthamoeba spp.: determining the role of the enzyme during the encystment process using RNA interference.

Lorenzo-Morales J., Klieščiková J., Martínez-Carretero E, De Pablos LM, Profotová B, Nohýnková E, Osuna A, Valladares B.

Eukaryotic Cell (2008), 7: 509-517 (IF=3.830)

Stress-induced pseudocyst formation--a newly identified mechanism of protection against organic solvents in acanthamoebae of the T4 genotype.

Klieščiková J., Kulda J., Nohýnkova E.

Protist (2011), 162: 58-69 (IF=3.136)

Propylene glycol and contact-lens solutions containing this diol induce pseudocyst formation in acanthamoebae.

Klieščiková J., Kulda J., Nohýnková E.

Experimental Parasitology (2011), 127: 326-328 (IF=2.122)

Encystation in Balamuthia mandrillaris – what we know about

Klieščiková J., Kulda J., Nohýnková E.

In preparation

The carbohydrate composition of *Acanthamoeba* cyst walls: first detection of β-1,3-1,4-linked glucan in protozoan organism.

Klieščiková J., Kulda J., Dupejová J., Šterba J., Řehulka, P., Nohýnková, E.

In preparation

RESULTS:

In my work I focused on the differentiation of two sister genera, *Acanthamoeba* and *Balamuthia*, into resistant stages with special emphasis on the CW assembly and the carbohydrate composition of the CW layers. Here, both published and unpublished results are presented.

ENCYSTATION

In order to study differentiation in *Acanthamoeba* spp., encystation medium according to Neff and Neff (1969) resembling the starvation conditions was employed. This medium enabled synchronized encystation as majority of *Acanthamoeba* trophozoites (up to 90%) exposed to this medium readily differentiated into mature cysts with double-layered CW within the first 24 hours in 37°C.

In order to follow the polysaccharides synthesis during encystation, intravital staining with Calcofluor white according to the method previously used (Choi and O`Day, 1983) was introduced. As mentioned in the introduction, Calcofluor white in higher concentration can lead to inhibition of cellulose synthesis (Mizuta and Brown, 1992), therefore low concentrations of 0.0005% not interfering with encystation were used.

The trophozoites induced to encyst remained in their ameboid form until 8th hour. However, already from the 6th hour Calcofluor white positive vesicles appeared in the cytoplasm of encystating amoebae. The number of trophozoites with the vesicles increased up to 8th hour, when the positive signal appeared on the plasma membrane of already rounded cells. After 10 hours of encystation, the exocyst synthesis started by appearance of tiny Calcofluor white positive dots covering the surface, which were increasing in size but omitting future ostioles. After 12 hours, majority of cells presented exocyst as a thin Calcofluor white positive layer covering the whole cell. Endocyst was synthesized during another 8-12 hours and was confirmed by the presence of cysts with both layers of the cyst wall after 24 hours post induction. At this stage, PI staining still displayed cytoplasm and nucleus, however after 48 hours post induction, the stain did not enter the cyst, most probably due to the firm layer of the endocyst which continued to be synthesized.

GOLGI PARTICIPATION IN THE CYST WALL ASSEMBLY

The first change observed in the encystating amoebae is the appearance of tiny Calcofluor white positive vesicles in the cytoplasm after 6 hours of induction of encystation. These seem

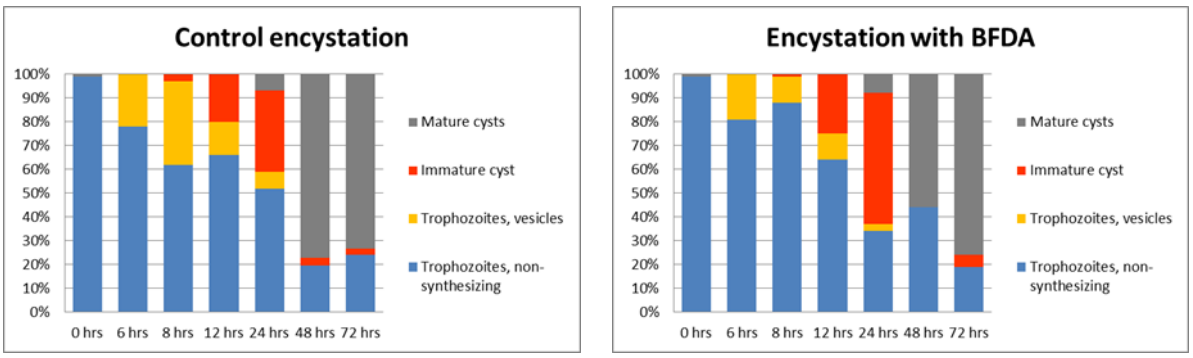
to be transported to the cell surface where they release their content and resemble encystation specific vesicles already described in *Entamoeba invadens* or *Giardia intestinalis* and also observed in *Acanthamoeba* spp. (Lujan et al., 1997; Chavez-Munguia et al., 2007).

To evaluate the possibility of the Golgi origin of these vesicles, we used BFDA, inhibitor of the organelle trafficking in concentrations of 10 or 20 μg/ml.

The cells were induced to encyst in the presence of Calcofluor white as described above. BFDA was added at 6th hour post induction for two hours. The number of cells with Calcolfluor positive vesicles was calculated before and after two hours of exposure when the medium was withdrawn and replaced by fresh encystation medium.

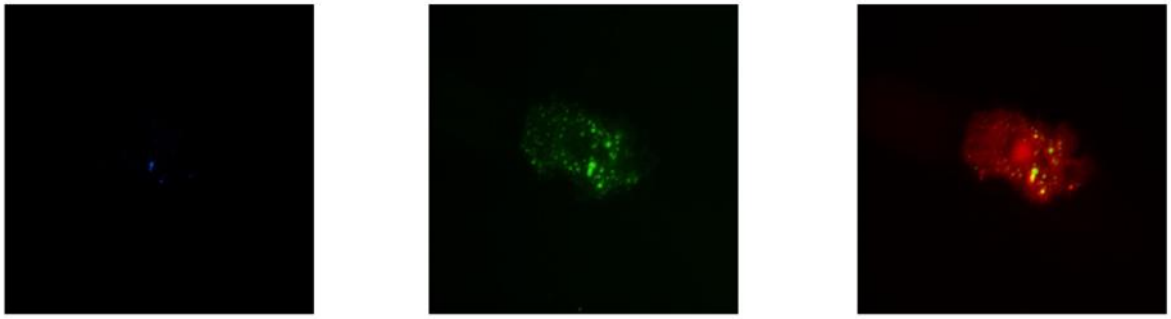
Exposure of encystating trophozoites to BFDA led to the decrease of the number of cells with Calcofluor white positive vesicles at 8th hour from 35% in the control to 11% in the treated cells (see graph). Moreover, the number of cells with positive vesicles, which increased in the control cells between the 6th and the 8th hour for 59%, decreased for 58% in the cells treated with BFDA. The course of encystation in the treated cells slowed down, but was not completely abolished.

We were unable to evaluate higher doses of BFDA, which were used to inhibit the encystation in *Giardia* (Lujan et al., 1995), due to the effect of solvent as described further below.



In order to further confirm the Golgi origin of the vesicles, co-staining experiments were done. Four specific markers of Golgi apparatus [antibody against 58kDa protein (Abcam), intravital probe CellLight Golgi RFP BacMam (Life Technologies), NBD C6 fluorescein conjugated ceramides (Invitrogen) and Wheat germ agglutinin WGA (Vector laboratories)] were evaluated in *Acanthamoeba* cells and control mammalian cells (Vero cell lines). All stains gave positive signal when employed on the Vero cells, but only WGA, conventional marker of trans Golgi network (Kanawaza et al., 2008) gave positive staining in

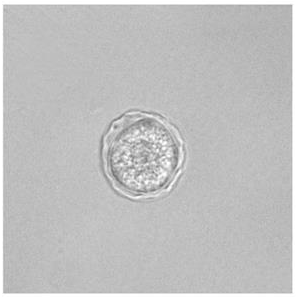
acanthamoebae. The WGA positive vesicles corresponded to the Calcofluor white positive encystation specific vesicles, further pointing out their Golgi origin.



Calcofluor white vesicles WGA vesicles Merge +PI

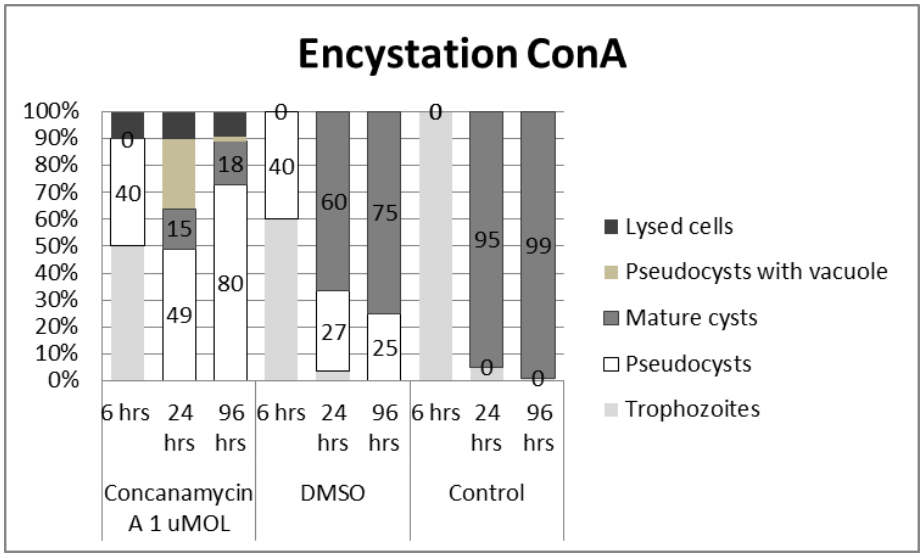
Studies detecting the content of the vesicles by employing specific antibodies detecting polysaccharides (cellulose, mixed-linkage glucan, β-mannan) in plants were inconclusive due to high background but the modification of the procedure might clarify whether and which material is transported by these vesicles to cell surface in the future studies.

In order to study the effect of increased lysosomal pH on the synthesis of polysaccharides during encystation in acanthamoebae, Concanamycin A (inhibitor of vacuolar H^+ATP-se) was employed in concentration of 1 μmol. In treated cells, none of the amoebae were capable of differentiating into mature cyst with double layered CWs within 24 hours of exposure as shown in the graph below. Based on this results it can be concluded, that correct acidification of the endomembranous system seems to be crucial for differentiation, but the mechanism by which the encystation is inhibited is unknown. The impact on the polysaccharide synthesis needs to be also further evaluated as the drug did not prevent the synthesis of pseudocyst coat (see below) containing polysaccharides with β-1,4-linkages as suggested by Calcofluor white staining. Formation of large vacuole in the treated cells resembled that already observed in *Dictyostelium discoideum* by Temesvari et al. (1996). However, further studies addressing the phenomenon needs to be conducted to explain the process in more detail.



Control encystation

Concanamycin A encystation



COMPOSITION OF THE MATURE CYST WALLS OF ACANTHAMOEBA

In order to characterize the carbohydrate composition of *Acanthamoeba* CW layers, antibodies against different polysaccharides already successfully used in plants were used (Meikle et al., 1994; McCartney et al., 2003; Tretheway et al, 2005).

The mature cysts of acanthamoebae had to be homogenized by Mini Bead Beater prior the staining, as the exocyst layer seemed to create mechanical barrier preventing the antibody binding. Lysis of the CW prior staining was already used in a study done by Linder et al (2002), where it was demonstrated that cellulose binding domain from *Trichoderma reesei* cellulase bind to *Acanthamoeba* CW only after 30 minutes digestion by trypsin. Moreover, the treatment with cellulase did not affect the morphology of the intact cyst, but only CWs obtained by previous homogenisation suggesting, that cellulose is major part of the inner CW, and the outer layer act as a barrier preventing the binding of the enzyme (Barrett and Alexander, 1977).

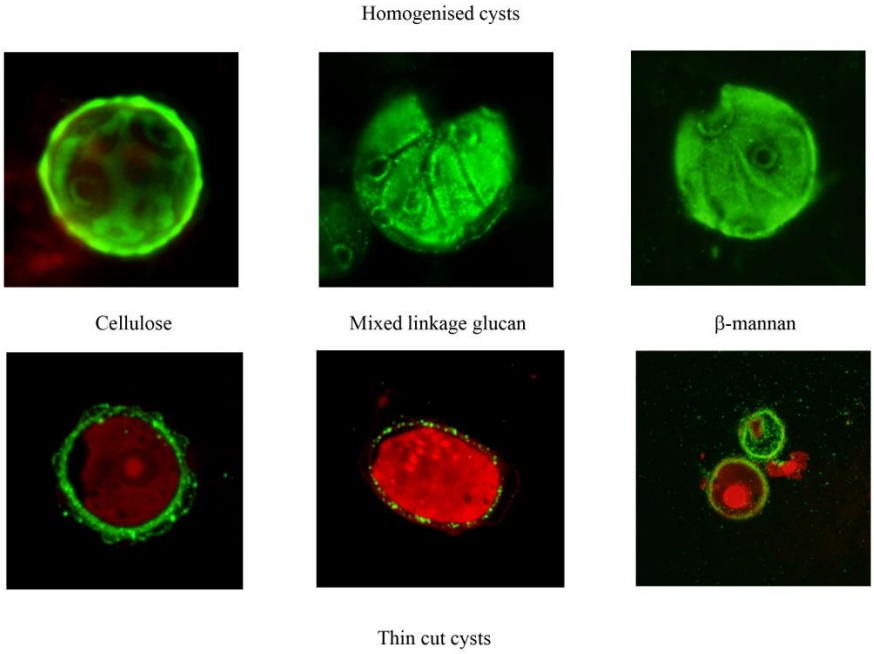
Based on the results we can conclude, that *Acanthamoeba* endocyst has got a very complex architecture. Cellulose (detected by CBM3a/Cellulose binding module), β-mannan and MLG

(both detected by specific antibodies) were detected in this layer. Whole appearance of the endocyst on fluorescence microscopy resembles a football ball. The ostiole can be easily distinguished as a rounded structure free of signal, inside of which a belt (operculum?) positive for all (antibodies and CBM3a) is found. Interestingly, lines free of signal leading from one ostiole to other were also detected in endocyst of acanthamoebae. It is tempting to hypothesize, that these are sites where endocyst and exocyst specifically connect. The staining with both antibodies and CBM3a give very similar appearance pointing out that endocyst has complex structure, where similarly to plants cellulose seems to be embedded in the matrix created by the other polysaccharides and possibly glycoproteins.

The specificity of the staining was confirmed by enzymatic digestion, when upon treatment of endocyst by licheninase (enzyme digesting specifically MLG) the positive signal of MLG disappeared. Similarly, endocysts treated with cellulase did not present positive signal when stained by CBM3a.

Immunogold electron microscopy of cyst further confirmed, that MLG and β-mannan are specifically present in the endocyst, whereas cellulose was detected in both, exocyst and endocyst layer although the amount seem much higher in the latter.

Based on the Maldi TOF/TOF analysis it seems that the MLG of acanthamoebae has got similar composition to that found in barley where the most abundant oligomer seem to have degree of polymerization 2 (DP2), followed by DP3 and DP4. Based on these results it seems that *Acanthamoeba* MLG consists primarily of cellobiose units, followed by cellotriose and cellotetraose units connected by single β-1,3-linkages.



CELLULOSE SYNTHESIS

Unlike the plants where minimum 3 different cellulose synthases create a functional terminal complex and *Dictyostelium discoideum* expressing a single gene, acanthamoebae during encystation express two cellulose synthases. Both seem to be expressed in two distinct phases: as early as 30 minutes post induction and later the expression increases again during 6th and 8th hour (Kliescikova et al., 2011a).

In several strains of acanthamoebae we were unable to detect by PCR the third gene proposed to be cellulose synthase according to Anderson et al. (2005). It could be possible, that only two genes encoding cellulose synthase are sufficient for the encystment in acanthamoebae and the role of the third proposed enzyme is so far unclear.

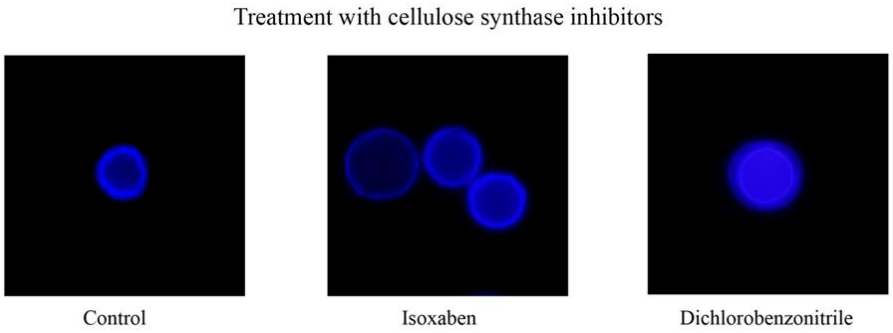
Two known inhibitors of cellulose synthesis in plants Isoxaben and Dichlorobenzonitrile were used to inhibit the synthesis and to study the impact on the encystation of *Acanthamoeba* spp.

The cells grown in PYG in 25°C were induced to encyst in EM Neff + 0.0005% Calcofluor white. The inhibitors in concentrations mentioned below were added to the encystation medium at time 0. The effect of the inhibitors was evaluated after 24 hours. When exposed to the lower concentrations (2 μmol/l and 20 μmol/l of Isoxaben and 1 μmol/l and 10 μmol/l of Dichlorobenzonitrile) approximately 50% of the cells proceeded with encystation similarly to the control cells and synthesized both layers of the CW, positive with Calcolfuor white staining suggesting that the synthesis of polysaccharides was not affected. The remaining cells formed pseudocysts (see below).

Based on these results it could be concluded that lower concentrations of inhibitors had no effect on the encystation of acanthamoebae whereas the effect of higher concentrations could not be evaluated due to the specific reaction of cells to the solvent as described below.

Our results correspond to those obtained in the study of Dudley et al. (2007) but our conclusions are different. The authors of the study concluded that the inhibition of cellulose synthesis by Dichlorobenzonitrile (in concentrations from 240 to 480 μmol/l) inhibited the encystation by 70%, as the treated cells exposed to 0.5% SDS lysed, whereas mature cysts were resistant. It was noted by the authors that dichlorobenzonitrile treatment did not abolish the fluorescence of Calcofluor white. The authors concluded that the positive signal remained due to the possible reaction of Calcofluor white with chitin or callose as this fluorescent brightener has got low specificity for cellulose. However, as they used methanol in high

concentrations to dissolve dichlorobenzonitrile, the effect might have been, similarly to our study, result of the solvent rather than the inhibitor itself.



THE SOURCE OF UDP-GLUCOSE FOR CELLULOSE SYNTHESIS

Cellulose, linear polymer of glucose connected by β-1,4-linkages is one of the major carbohydrates present in the CWs of acanthamoebae. Adequate substrate (UDP-glucose) was thought to be generated in acanthamoebae by two possible pathways: glyoxylate pathway via acetylCoA from lipidic sources or from the glycogen degradated by glycogen phosphorylase (as discussed in the introduction). Here I summarize the results of our work done in cooperation with University of La Laguna, Tenerife, Canary Islands and published in Eukaryotic Cell (Lorenzo-Morales et al., 2008).

In our study it was shown, that glycogen phosphorylase is in *Acanthamoeba* spp. regulated transcriptionally as the mRNA encoding this enzyme was specifically detected during encystation, but not in a trophozoite stage. Northern blot analyses revealed that the expression is limited to the cells in the early phase of encystment, between 8 and 24 h post stimulation. Western Blot analysis and zymogram assays detected glycogen phosphorylase and its activity at 12, 24, and 48 h after the induction of encystment.

In order to further characterize the role of glycogen phosphorylase for the encystment, specific siRNAs were designed in order to knock down the glycogen phosphorylase gene.

Transfection of encystating cells with these siRNAs resulted in complete silencing of the glycogen phosphorylase gene which was not detected by Northern or Western Blot analysis. Majority of acanthamoebae cells treated with siRNA were unable to complete the encystation process as observed by light, fluorescent and scanning electron microscopy. The course of encystation was similar in both treated and untreated cultures until 12^{th} hour post induction. Trophozoites in both cultures remained ameboid until approximately 10^{th} hour, then they rounded up and the exocyst deposition was detected in both cultures by intravital staining with Calcofluor white as described above. Untreated cultures continued with the

differentiation and at 72nd hour 83% of the population was represented by mature cysts with two layers of the CW. In siRNA treated cultures, even after 72 hours, 70% of the cells remained as immature precyst, with only the single outer layer of the CW was present. The addition of glucose to encysting cultures (both siRNA treated and control) had no effect on the cell phenotypes or proportions or the patterns of cellulose on the cell surfaces. Mature cysts (presenting both layers of the cyst wall after 72 h of encystment) were fully resistant to the treatment with 0.5% SDS, whereas single-walled precysts (observed mainly in the siRNA-treated cultures after 72 h) did not survive the exposure and underwent cell lysis.

The results point out the importance of glycogen phophorylase for the encystation, which might participate in glycogen degradation to glucose which seem to be further used for cellulose synthesis in the inner layer of the CW.

REACTION OF ACANTHAMOEBAE TO THE SOLVENTS: Pseudocyst formation

During inhibitory experiments (see above) we noticed a very specific reaction of acanthamoebae to organic solvents used to dissolve the inhibitors. Actually, this reaction prevented us from evaluating effect of higher concentrations of these inhibitors on encystation. Testing behaviour of acanthamoebae in the presence of organic solvents enabled us to describe a new type of *Acanthamoeba* differentiation leading to formation of a pseudocyst. Here I summarize results which were published in Protist (Kliescikova et al., 2011a) and Experimental Parasitology (Kliescikova et al., 2011b).

*Acanthamoeb*a trophozoites exposed to penetration enhancers such as methanol, acetone or DMSO at concentrations ranging from 0.1% to 10% promptly rounded up and detached from the surface. Within 2 h, a smooth, thin, single-layered Calcofluor white positive wall was visible on the surface of the rounded cyst like cells or pseudocysts. When the medium with solvent was exchanged for a fresh one, the pseudocysts promptly (within 2 h) transformed into amoeboid trophozoites leaving their coat behind and continued to multiply (in growth medium) or encysted (in encystation medium).

To prove that the pseudocyst does not represent an immature form of the acanthamoeba cyst but is a specific stage created during acute stress, further studies were conducted.

It was observed using transmission electron microscopy that the single-layered wall of the mature pseudocyst is composed of a finely fibrillar coat resembling glycocalyx that externally covers the plasma membrane. The first sign of the coat assembly, evident by loose discontinuously dispersed fibrils, was observed on the surface of already rounded cells with

finely wrinkled plasma membranes. The cytoplasm of these immature pseudocysts contained many mitochondria and lysosomes as well as prominent cisternae of RER. The coat material seems to be deposited on the surface through fusion of cytoplasmic tubules with the plasma membrane. In contrast to the texture of the pseudocyst coat, the amorphous ultrastructure of the early exocyst exhibits a markedly distinct pattern: it is composed of irregularly layered dense amorphous material deposited externally on the plasma membrane. The coat of the mature pseudocyst is composed solely of mannose/glucose moieties, whereas the exocyst contains mannose/glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine residues and mannose/glucose and N-acetylglucosamine were detected in the endocyst layer.

Moreover, CSP21, a cyst-specific protein and encystation-specific marker (Hirukawa et al. 1998) was not expressed at any time point during the pseudocyst formation. Moreover, we have confirmed that the protein was expressed exclusively during the encystation, with the highest expression seen at 24 h. Differentiation into both the cyst and the pseudocyst required the expression of two cellulose synthases, cellulose synthase I and II (CSI and CSII, respectively) and glycogen phosphorylase. However, timing and level of expression of the two synthases varied during differentiation. Pseudocyst coat protects the cell from heat up to 55°C, alkaline pH and desiccation.

Based on the results it can be concluded, that acanthamoebae differentiate into the mature cyst when chronic conditions such as changes in osmolarity or starvation threaten the cell survival. Encystation can be considered as relatively slow response as it requires minimum 24 hours to differentiate into mature cyst. However, if the cell is exposed to acute stress in the environment where its membrane integrity might be affected, it responds by pseudocyst formation. This reaction seems to be uniform among different genotypes and is not restricted to pathogenic strains.

In further experiments, propylene glycol, additive to contact lens solutions as ocular lubricant or osmotic agent was evaluated for the possibility of induction of pseudocyst formation in various genotypes of acanthmaoebae. It was confirmed that exposure of acanthamoeba trophozoites to this substance in concentration ranges from 0.25% to 10% indeed leads to formation of pseudocyst. Moreover, two contact lens solutions containing this diol (AMO Complete Moisture Plus and MeniCare Soft) induced the same reaction in all observed genotypes (from 45% in *A. castellanii* Neff up to 75% in the T5 genotype).

Complete Moisture Plus contact lens solution is infamously known for the outbreak of AK and had to be recalled from the market. As exposure of acanthamoebae to this solution leads to pseudocyst formation, it is tempting to speculate, that it were the pseudocysts which were

responsible for the infection of a human host and caused keratitis (Kliescikova et al., 2011a,b).

Taken together, pseudocyst formation in contact lens solutions may have a significant impact on the survival of amoebae: it may represent the source of trophozoites which are recruited when establishing the infection. As such, this may lead to an increased number of AK cases, although other factors contributing to the risk of the disease, such as lesser hygiene, have to be considered.

Differentiated cells resembling morphologically the pseudocyst were since our publication observed several times: as a response of *Acanthamoeba* trophozoites to chloramine (Mogoa et al., 2011), to the treatment with alkylphosphocholines (Lukac et al., 2012), as a response to exposure to the multipurpose contact lens solutions (Ahearn et al., 2012; Imayasu et al., 2013).

ENCYSTATION INDUCTION IN *BALAMUTHIA MANDRILLARIS*

To achieve synchronization of encystation in *B. mandrillaris*, different growth or encystation media described for acanthamoebae and their modifications (see the chart below) were evaluated.

The human isolate of *B. mandrillaris* (CDC V194) which readily encysts in tissue cultures using a Vero cell feeder layer did not respond by synchronous encystation to any stress conditions used in our study. Neither starvation represented by lack of nutrients, high pH, activation of cellulose synthase using Mg^{2+} or Ca^{2+} co-factors nor mechanical stress induce sufficient cyst formation. Moreover, the effect of osmotic stress (Siddiqui et al., 2010) was also not confirmed as only 8% glucose in DMEM but not in other media and no other monosaccharides stimulated the encystment.

Approximately 21% of trophozoites differentiated into mature cysts with three layers of the cyst wall as observed by light microscopy within 96 hours in DMEM supplemented with 8% glucose. However, careful examination by electron microscopy showed that these induced "cysts" did not share the same morphological characteristics with the spontaneously formed cysts. Only minority had three distinguishable layers of the cyst walls but thickness of the wall was significantly lesser than in the cysts formed during co-culture with Vero cells and except of the mesocyst, the wall layers were badly differentiated. Remaining "cysts" were rather rounded cells enveloped with layers of cyst wall material resembling immature cysts.

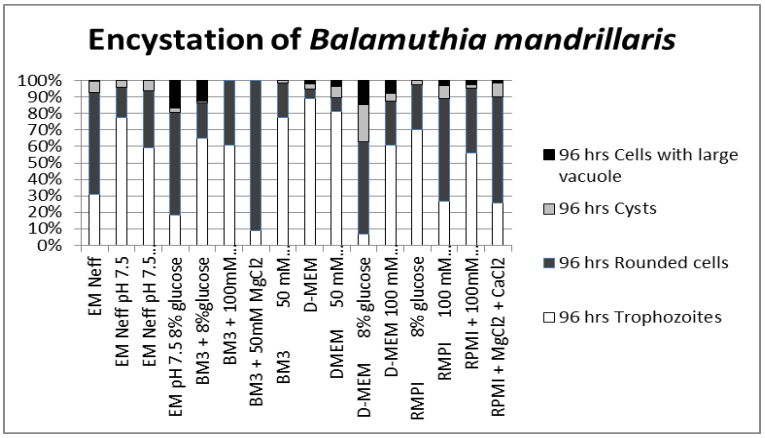
In the other 'encystation media' amoebae retracted their podia within first 48 hours, rounded up their cytoplasm becoming more dense (up to 65% of cells in EM Neff), however, no

further progress characterized by presence of visible CW layers was observed in the following 48 hours after which the experiment was terminated.

To summarize, none of the tested media produced adequate amount of the cysts within 96 hours after which the experiments were terminated (see graph below).

Our findings are in discordance with previous observations by Siddiqui et al (2010), who observed up to 70% encystation rate when 100 mM galactose was added to RPMI 1640 medium. When our strain of *Balamuthia* was exposed to the same conditions, we have observed that approximately 57% of the amoebae rounded up within 72 hours of exposure. Out of them 46% had a very dense cytoplasm resembling that of mature cyst, but no CWs with typical architecture were present. Only 5% mature cysts with triple layered CWs were observed when the experiment was terminated (72 hours). The encystation rate in our study was only 5% in repeated experiments in contrast to 70% observed in the study of Siddiqui et al. (2010). Interestingly, the percentage of rounded trophozoites in our study was more or less similar to the encystation rate in the mentioned study. The number of mature cysts versus rounded trophozoites in our study was evaluated manually by counting the appropriate cells under the light microscope whereas in the study of Siddiqui et al. total number of cysts was calculated by automated haemocytometer after lysis of all the present trophozoites by 0.5% SDS. No further morphological description of the induced cyst stage or photography is provided. It seems that evaluation of the encystation rate solely based on automatic haemocytometry might not be sufficient as without proper description of the morphology, rounded cells with dense cytoplasm might get mistaken for mature cysts. However, other explanations of the differences between these results have to be taken into consideration such as response variabilities between different strains and/or influence of the culture media, in which the amoeba was kept, similarly to that observed in acanthamoebae strains (Stratford and Griffiths, 1978; Shoff et al., 2007). Moreover, with regard to difficulties in *Balamuthia* encystation induction, it was already suggested, that in contrast to acanthamoebae which readily encyst in the axenic growth media the complexity of the BM-3 medium probably prevents balamuthias from encystation (Schuster and Visvesvara, 1996). Due to the inadequate encystation rates and observed differences between induced and spontaneously formed cysts, we have used only the spontaneously formed cysts in the further experiments focused on the composition of the *B. mandrillaris* CWs.

%		EM Neff	EM Neff pH 7.5	EM Neff pH 7.5 50 mM MgCl2	EM pH 7.5 8% glucose	BM3 + 8%glucose	BM3 + 100mM galactose	BM3 + 50mM MgCl2	BM3 50 mM MgCl2 0.4mM CaCl2	D-MEM	DMEM 50 mM MgCl2 0.4 mM CaCl2	D-MEM 8% glucose	D-MEM 100 mM galactose	RMPI 8% glucose	RMPI 100 mM galactose	RPMI + 100mM galactose	RPMI + 50 mM MgCl2 + 0.4 mMol CaCl2
96 hrs	Trophozoites	31%	77%	59%	19%	65%	61%	9%	78%	89%	81%	7%	61%	70%	27%	56%	26%
	Rounded cells	62%	18%	35%	62%	22%	39%	91%	21%	6%	8%	56%	27%	27%	63%	39%	65%
	Cysts	7%	4%	6%	2%	1%	0%	0%	2%	3%	7%	22%	5%	3%	8%	2%	9%
	Cells with large vacuole	0%	0%	0%	17%	13%	0%	0%	0%	2%	4%	15%	8%	0%	3%	2%	1%



CYST WALL COMPOSITION OF *BALAMUTHIA MANDRILLARIS*

Surprisingly, our study did not confirm the presence of cellulose in *Balamuthia* CW as suggested by Siddiqui et al. (2009).

When mature cysts were stained with Calcofluor white either intravitally or post fixation, no layer of the cyst wall exhibited positive signal, although some level of autofluorescence was noted (see the pictures below). In order to rule out the inaccessibility of cellulose to the stain, the cysts were homogenized prior the staining. Both intact cysts and cyst walls were then stained with Calcofluor white, immunostained using CBM3a (detecting cellulose and successfully used in acanthamoebae), antibody against MLG and β-mannan, and panel of fluorescein conjugated lectins by method already successfully used in acanthamoebae (Kliescikova et al., 2011a). None, Calcofluor white, antibodies or CBM3a detected carbohydrate structures in both, intact and homogenised *Balamuthia* CW layers. Moreover, no positive signal was detected when fluorescein conjugated lectins detecting glucose, mannose, galactose, N-acetylgalactosamine, N-acetylglucosamine, galactosyl-β-N-acetylgalactosamine, α/β-N-acetylgalactosamine were used.

Siddiqui et al. (2010) has shown that CWs of *Balamuthia* contain very little carbohydrates – less than 0.1%. Out of this, majority is represented by glucose (79%) and 21% of mannose of the dry weight. This result was quite surprising as usually, the amount of carbohydrates in the cyst stage of different protozoa is much higher: *Acanthamoeba* CW contains more than 0.7% carbohydrates, out of those 35% of cellulose (Dudley et al.2009, Neff and Neff, 1969) and there is more than 63% carbohydrate content in the *Giardia* cyst (Jarroll et., 1989). Therefore,

it is possible, that our methods were not sensitive enough to detect the low amount of polysaccharides in the CW of *Balamuthia*.

In order to investigate the possibility of proteins/glycoproteins composition of the CWs, the cysts were treated by proteases with different specificities for 24 hours: 1mg/ml pronase, 0.5 mg/ml trypsin, 0.5 mg/ml papain and 1 mg/ml pepsin. After 24 hours, we have observed that the CWs of balamuthias were completely dissolved when treated by pronase and papain, but remained intact when treated by pepsin and trypsin. In contrast to balamuthias, all *Acanthamoeba* cysts treated by the same proteases did not show any defects in the morphology.

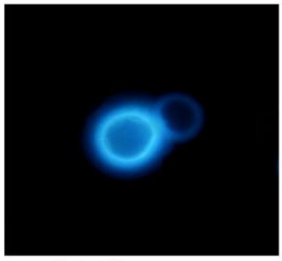
To evaluate the possibility of unmasking carbohydrate moieties after removal of the protein by protease treatment, balamuthias were treated for 12 hours with papain and pronase. Subsequently, the cysts were stained by fluorescein conjugated lectin. However, again no positive signal was obtained.

To evaluate whether the CW layers could be composed of lipids/glycolipids, the mature cysts of balamuthiae and acanthamoebae were stained by Nile Red 5 μg/ml in HEPES for 5 minutes. In both organisms, Nile Red positive material was found as small granules usually located in between exo- and endocyst (acanthamoebae) or in the mesocyst (balamuthiae). The number of granules ranged from 1-10 per cell and resembled cellular debris already described during acanthamoeba encystation.

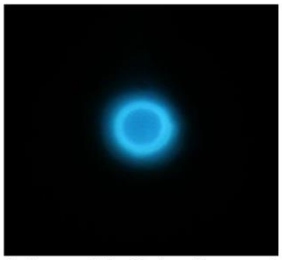
Based on theses preliminary results it seems, that *Balamuthia* CW is composed mainly of cysteine rich proteins. We were unable to detect any structures containing carbohydrates while employing various staining procedures. Lipids resembling cellular debris are found in the form of granules in the mesocysts.

In the future studies, the proteinaceous composition of the CWs of *Balamuthia* should be further evaluated and more studies are necessary to clarify the process of encystation in order to specifically address the differentiation of amoebae while treating the infections.

Cyst of *Balamuthia mandrillaris*

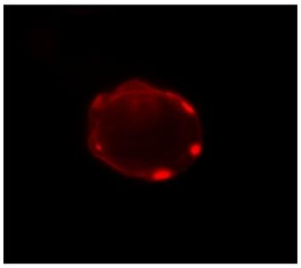


Autofluorescence

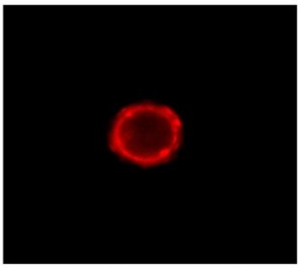


Staining with Calcofluor white

Nile Red staining of cysts

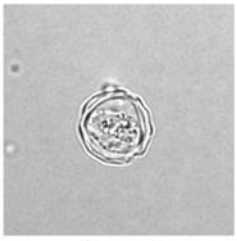


Acanthamoeba spp.



Balamuthia mandrillaris

Proteases treatment of Acanthamoeba cysts



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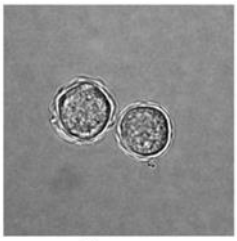
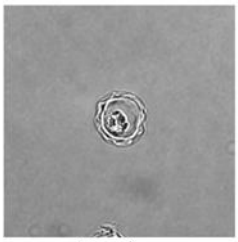


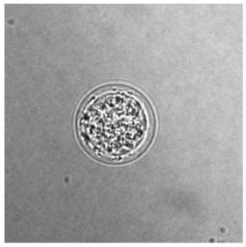
Pronase

Trypsine



Papain

Proteases treatment of Balamuthia cysts



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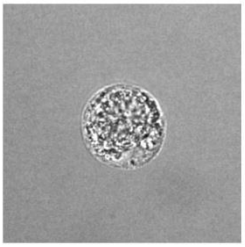

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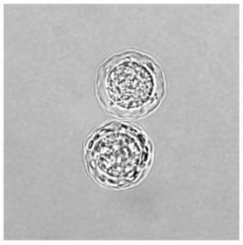
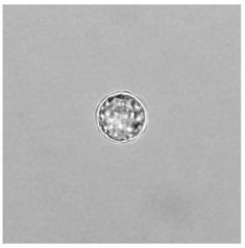


Trypsine



Papain

Summary of the results

1. *Acanthamoeba* pseudocyst formation

- Yet undescribed differentiation of *Acanthamoeba* spp. as a response to acute stresses of the environment threatening the survival of the cell by interfering with plasma membrane was described. In the so called ‚toxic' reaction the pseudocyst is formed within 2 hours of exposure to low concentrations of solvents such as methanol, DMSO, acetone or propylene glycol.

- Pseudocyst is basically different from mature cyst: rounded cell is covered by coat resembling glycocalyx in its ultrastructure, in which the only detected carbohydrate moiety, is glucose. The pseudocyst is resistant to alkaline pH and high temperatures.

- Pseudocyst formation seems to be of great medical importance in ophthalmology as contact lens solutions containing propylene glycol can induce this reaction in acanthamoebae.

2. *Acanthamoeba* encystation: Assembly of the cyst wall

- Encystation seems to be a response to chronic changes of the environment such as starvation, changes in temperature or pH which is not threatening the cell survival acutely. The whole process takes approximately 24 hours and results in a mature cyst with Calcofluor white positive double-layered cyst wall.

- During the first phases of *Acanthamoeba* encystation (6-8 hours after induction), tiny Calcofluor white positive vesicles containing cyst wall material are transported to the cell surface where they release their content. These vesicles seem to be of Golgi origin as suggested by inhibition by Brefeldin A and co-staining experiments.

- Brefeldin A in low concentration leads to decrease in the number of Calcofluor positive encystation specific vesicles. Exposure to this agent prolongs the time required to complete the encystation but is not able to stop the process entirely.

- Functional vacuolar ATPases maintaining intra Golgi pH seem to be essential for correct encystation as suggested by inhibition of encystation upon treatment with Concanamycin A, but the exact mechanism remains to be further investigated.

3. *Acanthamoeba* cyst wall composition and synthesis

- *Acanthamoeba* cyst wall has got complex carbohydrate composition with cellulose present in the exocyst and endocyst, whereas β-mannan and mixed linkage glucan are present solely in the endocyst .

- Two cellulose synthase genes are expressed de novo during encystation. Plant cellulose synthase inhibitors isoxaben and dichlorobenzonitrile do not seem to interfere with the synthesis in acanthamoebae suggesting other architecture of the synthase complexes (dichlorobenzonitrile) or differencies in the amino acid composition of the enzymes (Isoxaben).

- Glycogen is likely the main source of glucose for cellulose synthesis as silencing of glycogenphosphorylase by specific siRNAs prevents the endocyst assembly and blocks the encystation. Treated cells are more fragile and are lysed upon treatment with detergents unlike the mature cysts.

- Mixed-linkage glucan was detected in the *Acanthamoeba* cyst wall. The architecture of the mixed-linkage glucan regarding the number of oligosaccharides composition is similar to that of plants: containing high number of cellobiose, followed by cellotriosyl and small number of cellotetraosyl units connected by β-1,3-linkages. Detection of this „rare“polysaccharide in protozoan organism indicates that it might me more prevalent in the nature than previously thought.

4. *Balamuthia mandrillaris* encystation and cyst wall composition

- Any in vitro conditions represented by different encystation media promoting synchronised encystment in *Balamuthia mandrillaris* was not discovered.

- The cyst wall of *Balamuthia* seems to be mostly proteinaceous containing mostly cysteine-rich proteins. We were unable to detect any polysaccharides or carbohydrate moieties in the cyst wall in our study. Mesocyst seems to contain cellular debris such as lipidic granules.

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APENDIX

Original articles